

11/17/00
JCS

HAMILTON, BROOK, SMITH & REYNOLDS, P.C.

A

Box
SG
**UTILITY
PATENT APPLICATION
TRANSMITTAL**
*(Only for new nonprovisional applications under
37 C.F.R. 1.53(b))*

Attorney Docket No. 1242.1035-002

First Named Inventor or Application Identifier Leonard I. Zon

Express Mail Label No. EL552576675US

Title of Invention

FERROPORTIN1 NUCLEIC ACIDS AND PROTEINS

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

ADDRESS TO: Assistant Commissioner for Patents
Box Patent Application
Washington, D.C. 20231

1. Fee Transmittal Form
(Submit an original, and a duplicate for fee processing)

2. Specification **[Total Pages [87]]**
(preferred arrangement set forth below)

- Descriptive title of the invention
- Cross References to Related Applications
- Statement Regarding Fed sponsored R & D
- Reference to microfiche Appendix
- Background of the Invention
- Summary of the Invention
- Brief Description of the Drawings
- Detailed Description
- Claim(s)
- Abstract of the Disclosure

3. Drawing(s) (35 U.S.C. 113) **[Total Sheets [3]]**
 Formal Informal

4. Oath or Declaration/POA **[Total Pages []]**

- a. Newly executed (original or copy)
- b. Copy from a prior application (37 C.F.R. 1.63(d))
(for continuation/divisional with Box 17 completed)
[NOTE Box 5 below]
 - i. DELETION OF INVENTOR(S)
Signed statement attached deleting inventor(s) named in the prior application, see 37 C.F.R. 1.63(d)(2) and 1.33(b).

5. Incorporation By Reference *(useable if Box 4b is checked)*
The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

6. Microfiche Computer Program *(Appendix)*

7. Nucleotide and/or Amino Acid Sequence Submission
(if applicable, all necessary)

- a. Computer Readable Copy
- b. Paper Copy *(identical to computer copy)*
[19] Pages
- c. Statement verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

8. Assignment Papers (cover sheet & documents)

9. 37 C.F.R. 3.73(b) Statement Power of Attorney
(when there is an assignee)

10. English Translation Document *(if applicable)*

11. Information Disclosure Statement (IDS)/PTO-1449 Copies of IDS Citations

12. Preliminary Amendment

13. Return Receipt Postcard (MPEP 503)
(Should be specifically itemized)

14. Small Entity Statement(s) Statement filed in prior application, status still proper and desired

15. Certified Copy of Priority Document(s)
(if foreign priority is claimed)

16. Other: _____

17. If a **CONTINUING APPLICATION**, check appropriate box and supply the requisite information:
 Continuation Divisional Continuation-in-part (CIP) of prior application No.: 09/567,672

Prior application information: Examiner: Unassigned Group Art Unit: 1633

18. CORRESPONDENCE ADDRESS

NAME	Doreen M. Hogle, Esq. HAMILTON, BROOK, SMITH & REYNOLDS, P.C.				
ADDRESS	Two Militia Drive				
CITY	Lexington	STATE	MA	ZIP CODE	02421-4799
COUNTRY	USA	TELEPHONE	(781) 861-6240	FAX	(781) 861-9540

Signature	Carol A. Egner	Date	November 17, 2000
Submitted by Typed or Printed Name	Carol A. Egner	Reg. Number	38,866

-1-

Date: 11/17/00 Express Mail Label No. EL 552576675 US

Inventors: Leonard I. Zon and Adriana Donovan

Attorney's Docket No.: 1242.1035-002

FERROPORTIN1 NUCLEIC ACIDS AND PROTEINS

RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Patent Application No. 09/567,672, filed May 9, 2000, which claims the benefit of U.S. Provisional Application No. 60/133,382, filed May 10, 1999. The entire teachings of both of these applications are hereby incorporated herein by reference.

GOVERNMENT SUPPORT

The invention was supported, in whole or in part, by a grant R01 DL53298-02 from the National Institutes of Health. The Government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Defects in iron absorption and utilization lead to iron deficiency and overload disorders. Adult mammals absorb iron through the duodenum, whereas embryos obtain iron through placental transport. Iron uptake from the intestinal lumen through the apical surface of polarized duodenal enterocytes is mediated by the divalent metal transporter, DMT1 (Fleming, M.D., *et al.*, *Nature Genet.*, 16:383-386, 1997; Gunshin, H., *et al.*, *Nature*, 388:482-488, 1997; Andrews, N.C., *N. Engl. J. Med.*, 334:1986-1995, 1996). A second transporter has been postulated to export iron across the basolateral

surface to the circulation. The function of this iron transporting protein may be perturbed in mammalian disorders of iron deficiency or overload. Drugs to alter the function of this iron transporting protein may be useful to treat such diseases as hemochromatosis and some forms of anemia.

5 SUMMARY OF THE INVENTION

The invention relates to a number of nucleic acids, wherein the nucleic acids have SEQ ID NO:1, 3, 5 or 7 as described herein or the nucleic acids have nucleotide sequences related to those given specifically by SEQ ID NO by properties of hybridization, or by varying extents of identity, or by varying degrees of similarity as 10 can be determined by a computer program designed for the purpose of comparing nucleotide or amino acid sequences. SEQ ID NO:1 is the nucleotide sequence of a cDNA encoding a zebrafish ferroportin1; SEQ ID NO:3 is the nucleotide sequence of a cDNA encoding a mouse ferroportin1; SEQ ID NO:5 is the nucleotide sequence of a cDNA encoding a human ferroportin1. SEQ ID NO:7 is the nucleotide sequence of a 15 genomic DNA comprising the introns and exons of a human ferroportin1 gene. Also part of the invention are contiguous portions of any of the above nucleic acids, nucleic acids encoding any of the amino acid sequences described herein and nucleic acids encoding polypeptides which are variants of the Ferroportin1 proteins described herein by amino acid sequence. Further nucleic acids which are part of the invention are those 20 encoding a fusion polypeptide comprising a Ferroportin1 or a portion of a Ferroportin1.

Related to the isolated nucleic acids are vectors and host cells comprising nucleotide sequences identical to the isolated nucleic acids of the invention. In some cases, regulatory sequences can be operably linked to coding regions to allow expression of a gene. Such cells can be maintained under conditions in which the gene 25 is expressed and the encoded polypeptide is produced. The polypeptide can be purified by one or more steps to increase the proportion of polypeptide in the milieu of medium and material of cellular origin, thereby producing isolated polypeptide.

The term *regulatory element* refers to a genetic element which controls some aspect of the expression of nucleic acid sequences. For example, a promoter is a regulatory element which facilitates the initiation of transcription of an operably linked coding region. Other regulatory elements are splicing signals, polyadenylation signals, 5 termination signals and enhancers, for instance.

The invention also includes Ferroportin1 proteins, for example, those having amino acid sequence SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6, proteins which are naturally occurring mutants or variants of those proteins characterized by those specific amino acid sequences, and mutants and variants of those proteins identified as having 10 the specific amino acid sequence SEQ ID NO:2, 4, or 6 that are produced by laboratory manipulations of the nucleic acids encoding a Ferroportin1. Also within the invention are contiguous portions of any of the polypeptides with SEQ ID NO:2, 4, or 6, or portions of such mutants or variants of the polypeptides described herein as containing amino acid substitutions, or described herein as having a certain percent identity or 15 similarity to another sequence in a comparison. A further embodiment of the invention is a fusion polypeptide, which can comprise a Ferroportin1 of full-length amino acid sequence, as in SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6, or a contiguous portion thereof, or can comprise any of the mutants or variant polypeptides, or portions thereof, as described herein, for example by their amino acid sequence identity or similarity to 20 an amino acid sequence identified by SEQ ID NO, or by their activity (e.g., iron transport function) or property of binding to antibodies produced by immunizing an animal with a Ferroportin1.

Antibodies that bind to one or more Ferroportin1 proteins are also an aspect of the invention. Antibodies to a Ferroportin1 of one or more species can be produced, for 25 example, by introducing into an animal which is not the source of the Ferroportin1 immunogen a Ferroportin1 or an immunogenic portion thereof in a suitable medium, which can include such substances as stabilizing agents and adjuvant. Other known

methods can be used to make hybridomas producing monoclonal antibodies that bind to one or more Ferroportin1 proteins, as isolated, or as they exist in a cell membrane.

Other aspects of the invention include methods for identifying agents which bind to a Ferroportin1 (or to a mutant, variant, Ferroportin1 fusion protein or a contiguous portion of any of the foregoing) by steps that include contacting the agent with the isolated protein under conditions appropriate for binding of the agent to the isolated protein, and detecting a resulting agent-protein complex. Similar methods can be used to identify an agent which is an inhibitor or an enhancer of a function of a Ferroportin1 protein, where the steps can be the following: (a) combining (1) said isolated protein; 5 (2) the ligand of said protein; and (3) a candidate agent to be assessed for its ability to inhibit interaction between said protein of (1) and the ligand of (2), under conditions appropriate for interaction between the said protein of (1) and the ligand of (2); (b) determining the extent to which said protein of (1) and the ligand of (2) interact; and (c) comparing the extent determined in (b) with the extent to which interaction of said 10 protein of (1) and the ligand of (2) occurs in the absence of the candidate agent to be assessed and under the same conditions appropriate for interaction of said protein of (1) with the ligand of (2); wherein if the extent to which interaction of said protein of (1) and the ligand of (2) occurs is less in the presence of the candidate agent than in the absence of the candidate agent, the candidate agent is an agent which inhibits interaction 15 between said protein and the ligand of said protein. If greater export of the iron from the test cells compared to export of the iron from the control cells is observed, this is indicative that the agent is an enhancer of iron export by said protein. An agent can be further tested for its effect on a Ferroportin1 protein in an animal, if the following steps are carried out: a) administering the agent to one or more test animals; b) measuring 20 exogenously supplied iron in one or more samples of tissue or bodily fluid from said test animals; c) measuring exogenously supplied iron in one or more comparable samples of tissue or bodily fluid from suitable control animals; and d) comparing the iron of b) with the iron of c); whereby, lower iron in step b) than in step c) is indicative that the agent is 25

an inhibitor of said protein. An inhibitor of the iron transport function of a human Ferroportin1 can be used in a method for treating hemochromatosis in a human, said method comprising administering to the human an inhibitor of Ferroportin1 iron transport function, or such inhibitor can be used in a method for treating a disease or 5 medical disorder resulting from oxidative damage in a mammal, said method comprising administering to the mammal an inhibitor of Ferroportin1 iron transport function. Enhancers of a Ferroportin1 can be used in a method for treating iron deficiency anemia in a mammal, said method comprising administering to the mammal an enhancer of Ferroportin1 iron transport function.

10

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a map of the *weh* locus showing positional cloning of the *weissherbst* gene. The *weh* locus is depicted by a thick black bar just distal to the AFLP marker I36. Below the map is an enlarged view of the *weh* locus that depicts the BAC and PAC 15 genomic clones identified by a chromosomal walk in an analysis of 3873 meioses. Genotyping of a total of 1783 meioses from haploid animals and 2090 meioses from diploid mutants narrowed the critical interval containing the gene to the PAC clones 211O13 and 170G3. The numbers of recombination events identified on the proximal side (circles) and distal side (squares) of the *weh* locus are indicated.

20

Figure 2 is an amino acid sequence alignment of zebrafish, human and mouse Ferroportin1 (FPN1). The initiator methionine in all three species was established by the presence of upstream, in-frame stop codons. Shading indicates identical amino acids. Bars under sequence indicate predicted transmembrane domains. The mutations identified in the *weh*^{tp85c} and *weh*^{th238} alleles are indicated by black circles below the 25 affected amino acids.

Figure 3 is a bar graph showing the measurement of iron efflux from *Xenopus* oocytes. Oocytes expressing either DMT1 alone or DMT1 and FPN1 were loaded with ⁵⁵Fe by incubation in uptake buffer containing 60 μ M ⁵⁵FeCl₂. Efflux from individual

oocytes was measured by incubation of oocytes in 500 μ l of efflux buffer with or without 20 mg/ml apo-transferrin (- apoTfr and + apoTfr). After efflux, the total ^{55}Fe content of both the efflux solution and the individual oocytes was measured by scintillation counting. The data are expressed as an average (n=6) of the ratio of the 5 pmols of efflux to the pmols of uptake per oocyte.

DETAILED DESCRIPTION OF THE INVENTION

Iron is required for many cellular processes, but it can also be toxic when present in excess. Thus, iron homeostasis must be strictly maintained. Studies described herein employed zebrafish genetics to identify the multiple-transmembrane 10 domain protein Ferroportin1, an iron export protein. In the mammalian yolk sac and placenta, Ferroportin1 may play an important conserved role in the transport of iron from the maternal to the embryonic circulation. In adults, Ferroportin1 is likely to function in iron transport at the basolateral surface of duodenal enterocytes. In disorders such as iron deficiency or overload, tissues respond by altering normal iron utilization. 15 Ferroportin1 could be involved in the pathophysiology of iron deficiency anemias or iron overload syndromes, such as hemochromatosis.

As described herein, Ferroportin1 refers to an evolutionarily conserved family of proteins that mediate the transport of iron out of cells. The family includes proteins which are conserved at least as widely as from zebrafish to humans and exhibit very 20 different expression patterns in tissues. Specific embodiments described include Ferroportin1 proteins from mice, humans and zebrafish which have been shown to be functional iron transporters. The term Ferroportin1 can refer to other proteins sharing at least about 70% sequence similarity, more preferably at least about 80% sequence similarity, and still more preferably, at least about 90% sequence similarity, and most 25 preferably, at least about 95% sequence similarity.

One aspect of the invention relates to isolated nucleic acids or polynucleotides that encode a Ferroportin1 as described herein, such as those Ferroportin1 proteins

having an amino acid sequence SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6 and nucleic acids closely related thereto as described herein.

Using the information provided herein, such as a nucleic acid sequence set forth in SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5, a nucleic acid of the invention 5 encoding a Ferroportin1 polypeptide may be obtained using standard cloning and screening methods, such as those for cloning and sequencing cDNA library fragments, followed by obtaining a full length clone. For example, to obtain a nucleic acid of the invention, a library of clones of cDNA of a species of animal can be probed with a labeled oligonucleotide, such as a radiolabeled oligonucleotide, preferably about 17 10 nucleotides or longer, derived from a partial sequence. Clones carrying DNA identical to that of the probe can then be distinguished using stringent (also, "high stringency") hybridization conditions. By sequencing the individual clones thus identified with sequencing primers designed from the original sequence it is then possible to extend the sequence in both directions to determine the full length sequence. Suitable techniques 15 are described, for example, in *Current Protocols in Molecular Biology* (F.M. Ausubel *et al.*, eds.), containing supplements through Supplement 49, 2000, John Wiley and Sons, Inc., especially chapters 5, 6 and 7.

Embodiments of the invention include isolated nucleic acid molecules comprising any of the following nucleotide sequences: 1.) a nucleotide sequence which 20 encodes a protein comprising the amino acid sequence of human Ferroportin1 (SEQ ID NO:6), the amino acid sequence of mouse Ferroportin1 (SEQ ID NO:4), or the amino acid sequence of zebrafish Ferroportin1 (SEQ ID NO:2); 2.) nucleotide sequences of human *ferroportin1*, mouse *ferroportin1*, or zebrafish *ferroportin1*; 3.) a nucleotide sequence which is complementary to the nucleotide sequence of human *ferroportin1* 25 (SEQ ID NO:5), mouse *ferroportin1* (SEQ ID NO:3), zebrafish *ferroportin1* (SEQ ID NO:1); 4.) a nucleotide sequence which consists of the coding region of human *ferroportin1* (within SEQ ID NO:5), the coding region of mouse *ferroportin1* (within SEQ ID NO:3), or the coding region of zebrafish *ferroportin1* (within SEQ ID NO:1).

The invention further relates to nucleic acids (nucleic acid molecules or polynucleotides) having nucleotide sequences identical over their entire length to SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO: 5. It further relates to DNA, which due to the degeneracy of the genetic code, encodes a Ferroportin1 protein whose amino acid sequence is provided herein. Also provided by the invention are nucleic acids having the coding sequences for the mature polypeptides or fragments in reading frame with other coding sequences, such as those encoding a leader or secretory sequence, a pre-, or pro- or prepro- protein sequence. The nucleic acids of the invention encompass nucleic acids that include a single continuous region or discontinuous regions encoding the polypeptide, together with additional regions, that may also contain coding or non-coding sequences. The nucleic acids may also contain non-coding sequences, including, for example, but not limited to, non-coding 5' and 3' sequences, such as the transcribed, non-translated sequences, termination signals, ribosome binding sites, sequences that stabilize mRNA, introns, polyadenylation signals, and additional coding sequences which encode additional amino acids.

The nucleic acid molecules of the invention can comprise, in addition to sequences identified by SEQ ID NO or sequences related to these by variations and by hybridization as described herein, other sequences encoding unrelated (heterologous -- that is, with insignificant sequence similarity to a Ferroportin1) polypeptides or peptides. These peptides or polypeptides can be whole proteins, as occur naturally or as have been modified by design. Together, the nucleic acid sequences make up genes for hybrid or fusion proteins. For example, an unrelated marker sequence that facilitates purification (e.g., by affinity column) of the fused polypeptide can be encoded. In certain embodiments of the invention, the marker sequence can be a hexa-histidine peptide, as provided in the pQE vector (Qiagen, Inc.) and described in Gentz *et al.*, *Proc. Natl. Acad. Sci. USA* 86: 821-824 (1989), or an HA tag (Wilson *et al.*, *Cell* 37: 767 (1984)), or a sequence encoding glutathione S-transferase of *Schistosoma japonicum* (vectors available from Pharmacia; see Smith, D.B. and Johnson K.S., *Gene*

67:31 (1988) and Kaelin, W.G. *et al.*, *Cell* 70:351 (1992)). For additional applications, the unrelated nucleic acid sequence can encode a peptide or polypeptide which is immunogenic or which enhances the immunogenicity of the fusion protein or polypeptide. Nucleic acids of the invention also include, but are not limited to, nucleic acids comprising a structural gene and its naturally associated sequences that control gene expression.

The invention further relates to variants, including naturally-occurring allelic variants, of those nucleic acids described specifically herein by DNA sequence, that encode variants of such polypeptides as those having the amino acid sequences SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6. Such variants include nucleic acids encoding variants of the above-listed amino acid sequences, wherein those variants have several, such as 5 to 10, 1 to 5, or 3, 2 or 1 amino acids substituted, deleted, or added, in any combination. Variants include polynucleotides encoding polypeptides with at least 95% but less than 100% amino acid sequence identity to the polypeptides described herein by amino acid sequence. Variant polynucleotides hybridize, under low to high stringency conditions, to the alleles described specifically herein by DNA sequence. In one embodiment, variants have silent substitutions, additions and deletions that do not alter the properties and activities of the Ferroportin1. Allelic variants of the polynucleotides encoding human Ferroportin1 (SEQ ID NO:5), mouse Ferroportin1 (SEQ ID NO:3), and zebrafish Ferroportin1 (SEQ ID NO:1) will be identified as mapping to chromosomal locations corresponding to the chromosomal locations of the wild type genes.

Orthologous genes are gene loci in different species that are sufficiently similar to each other in their nucleotide sequences to suggest that they originated from a common ancestral gene. Orthologous genes arise when a lineage splits into two species, rather than when a gene is duplicated within a genome. Proteins that are orthologs are encoded by genes of two different species, wherein the genes are said to be orthologous.

The invention further relates to polynucleotides encoding polypeptides which are orthologous to those polypeptides having a specific amino acid sequence described

herein, such as the amino acid sequences (SEQ ID NO:2), (SEQ ID NO:4), and (SEQ ID NO:6). These polynucleotides, which can be called ortholog polynucleotides, encode orthologous polypeptides that can range in amino acid sequence identity to a reference amino acid sequence described herein, from about 65% to less than 100%, but

5 preferably 70% to 80%, more preferably 80% to 90%, and still more preferably 90% to less than 100%. Orthologous polypeptides can also be those polypeptides that range in amino acid sequence similarity to a reference amino acid sequence described herein from about 75% to 100%. The ortholog polynucleotides encode polypeptides that have similar functional characteristics (e.g., iron transport activity) and similar tissue

10 distribution, as appropriate to the organism from which the ortholog polynucleotides can be isolated.

Ortholog polynucleotides can be isolated from (e.g., by cloning or nucleic acid amplification methods) a great number of species, as shown by the sample of Ferroportin1 proteins from evolutionarily divergent species described herein. Ortholog 15 polynucleotides corresponding to SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5 are those which can be isolated from mammals such as rat, dog, chimpanzee, monkey, baboon, pig, rabbit and guinea pig, for example.

Further variants that are fragments of the nucleic acids of the invention may be used to synthesize full-length nucleic acids of the invention, such as by use as primers in 20 a polymerase chain reaction. As used herein, the term primer refers to a single-stranded oligonucleotide which acts as a point of initiation of template-directed DNA synthesis under appropriate conditions (e.g., in the presence of four different nucleoside triphosphates and an agent for polymerization, such as DNA or RNA polymerase or reverse transcriptase) in an appropriate buffer and at a suitable temperature. The 25 appropriate length of a primer depends on the intended use of the primer, but typically ranges from 15 to 30 nucleotides. Short primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with the template. A primer need not reflect the exact sequence of the template, but must be sufficiently

complementary to hybridize with a template. The term primer site refers to the area of the target DNA to which a primer hybridizes. The term primer pair refers to a set of primers including a 5' (upstream) primer that hybridizes with the 5' end of the DNA sequence to be amplified and a 3' (downstream) primer that hybridizes with the 5 complement of the 3' end of the sequence to be amplified.

Further embodiments of the invention are nucleic acids that are at least 80% identical over their entire length to a nucleic acid described herein, for example a nucleic acid having the nucleotide sequence in SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5. Additional embodiments are nucleic acids, and the complements of such 10 nucleic acids, having at least 90% nucleotide sequence identity to the above-described sequences, and nucleic acids having at least 95% nucleotide sequence identity. In preferred embodiments, DNA of the present invention has 97% nucleotide sequence identity, 98% nucleotide sequence identity, or at least 99% nucleotide sequence identity with the DNA whose sequences are presented herein.

15 Other embodiments of the invention are nucleic acids that are at least 80% identical in nucleotide sequence to a nucleic acid encoding a polypeptide having an amino acid sequence as set forth in SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6, and nucleic acids that are complementary to such nucleic acids. Specific embodiments are nucleic acids having at least 90% nucleotide sequence identity to a nucleic acid 20 encoding a polypeptide having an amino acid sequence as described in the list above, nucleic acids having at least 95% sequence identity, and nucleic acids having at least 97% sequence identity. Also included in the invention are nucleic acid molecules comprising at least 80%, 90%, 95%, or 97% of the coding region of any of SEQ ID NOs 1, 3 or 5.

25 The terms "complementary" or "complementarity" as used herein, refer to the natural binding of polynucleotides under permissive salt and temperature conditions by base-pairing. Complementarity between two single-stranded molecules may be "partial" in which only some of the nucleic acids bind, or it may be complete when total

complementarity exists between the single-stranded molecules (that is, when A-T and G-C base pairing is 100% complete). The degree of complementarity between nucleic acid strands has significant effects on the efficiency and strength of hybridization between nucleic acid strands. This is of particular importance in amplification reactions, which depend on binding between nucleic acid strands.

The invention further includes nucleic acids that hybridize to the above-described nucleic acids, especially those nucleic acids that hybridize under stringent hybridization conditions. Preferred nucleic acid molecules meeting these hybridization criteria also encode a polypeptide having an iron transport function. "Stringent hybridization conditions" or "high stringency conditions" generally occur within a range from about T_m minus 5°C (5° C below the strand dissociation temperature or melting temperature (T_m) of the probe nucleic acid molecule) to about 20° C to 25° C below T_m . As will be understood by those of skill in the art, the stringency of hybridization may be altered in order to identify or detect molecules having identical or related polynucleotide sequences. An example of high stringency hybridization follows. Hybridization solution is (6x SSC/10 mM EDTA/0.5% SDS/5x Denhardt's solution/100 µg/ml sheared and denatured salmon sperm DNA). Hybridization is at 64-65°C for 16 hours. The hybridized blot is washed two times with 2x SSC/0.5% SDS solution at room temperature for 15 minutes each, and two times with 0.2x SSC/0.5% SDS at 65°C, for one hour each. Further examples of high stringency conditions can be found on pages 2.10.1-2.10.16 (see particularly 2.10.8-11) and pages 6.3.1-6 in *Current Protocols in Molecular Biology* (Ausubel, F.M. *et al.*, eds., containing supplements up through Supplement 49, 2000). Examples of high, medium, and low stringency conditions can be found on pages 36 and 37 of WO 98/40404, which are incorporated herein by reference.

The invention further relates to nucleic acids obtainable by screening an appropriate library with a probe having a nucleotide sequence such as that set forth in SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5, or a probe which consists of the

coding region of any of these SEQ ID NOs, or a probe which is a sufficiently long fragment of any of the above; and isolating the nucleic acid. Such probes generally can comprise at least 15 nucleotides. Nucleic acids obtainable by such screenings may include RNAs, cDNAs and genomic DNA, for example, encoding iron transport

5 proteins of the Ferroportin1 protein family described herein.

Other nucleic acid embodiments are those comprising a nucleotide sequence encoding a contiguous portion of a polypeptide represented as having amino acid sequence SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6, wherein the portion is at least about 15 amino acids long, but can alternatively be at least 30 amino acids long or 60

10 amino acids long. The portion can be derived from amino acid sequence at the N-terminal, C-terminal or internal regions of SEQ ID NOs 2, 4 or 6.

Further uses for the nucleic acid molecules of the invention, whether encoding a full-length Ferroportin1 protein or whether comprising a contiguous portion of a nucleic acid molecule such as one given in SEQ ID NO:1, 3 or 5, include use as markers for

15 tissues in which the encoded protein is preferentially expressed (to identify constitutively expressed proteins or proteins produced at a particular stage of tissue differentiation or stage of development of a disease state); as molecular weight markers on southern gels; as chromosome markers or tags (when labeled, for example with biotin, a radioactive label or a fluorescent label) to identify chromosomes or to map

20 related gene positions; to compare with endogenous DNA sequences in a mammal to identify potential genetic disorders; as probes to hybridize and thus identify, related DNA sequences; as a source of information to derive PCR primers for genetic fingerprinting; as a probe to "subtract-out" known sequences in the process of

25 discovering other novel nucleic acid molecules; for selecting and making oligomers for attachment to a "gene chip" or other support, to be used, for example, for examination of expression patterns in embryonic development or in organs of an animal at a particular developmental stage.

Further methods to obtain nucleic acids encoding Ferroportin1 proteins include PCR and variations thereof (e.g., "RACE" PCR and semi-specific PCR methods). Portions of the nucleic acids having a nucleotide sequence set forth in SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5, (especially "flanking sequences" on either side of a 5 coding region) can be used as primers in methods using the polymerase chain reaction, to produce DNA from an appropriate template nucleic acid.

Once a fragment of the *ferroportin1* gene is generated by PCR, it can be sequenced, and the sequence of the product can be compared to other DNA sequences, for example, by using the BLAST Network Service at the National Center for 10 Biotechnology Information. The boundaries of the open reading frame can then be identified using semi-specific PCR or other suitable methods such as library screening. Once the 5' initiator methionine codon and the 3' stop codon have been identified, a PCR product encoding the full-length gene can be generated using cDNA as a template (the cDNA being generated from mRNA), with primers complementary to the extreme 15 5' and 3' ends of the gene or to their flanking sequences. The full-length genes can then be cloned into expression vectors for the production of functional proteins.

In some embodiments of the invention, the nucleic acid molecules can be modified at the base moiety, sugar moiety or phosphate backbone to change the stability, hybridization or solubility properties of the molecules. For example, the 20 deoxyribose phosphate backbone of the nucleic acids can be modified to generate peptide nucleic acids, or PNAs (Hyrup *et al.*, *Bioorganic and Medicinal Chemistry* 4 :5-23, 1996). PNAs are nucleic acid mimics in which the deoxyribose phosphate backbone is replaced by a pseudopeptide backbone and only the four natural nucleobases are retained. The neutral backbone of PNAs has been shown to allow for specific 25 hybridization to DNA and RNA under conditions of low ionic strength. The synthesis of PNA oligomers can be performed using standard solid phase peptide synthesis protocols as described by Hyrup *et al.* (1996) and Perry-O'Keefe *et al.*, *Proc. Natl. Acad. Sci. USA* 93:14670-14675, 1996. PNAs can be used in place of nucleic acids for

some applications, for example, as probes or primers for DNA sequence analysis and hybridization., or as antisense agents for sequence-specific modulation of gene expression.

Nucleic acid molecules of the present invention can be incorporated into various constructs (e.g., plasmids, bacteriophages, viruses, artificial chromosomes) and incorporated into host cells in these constructs or in one or more chromosomes of the host cell, for example, for further manipulation of the sequences or for production of an encoded polypeptide under suitable conditions for the growth or maintenance of the cells.

10 A host cell is a cell, or a descendant thereof, which has been transfected by an exogenous DNA sequence using methods within the skill of those in the art. See, e.g., Graham *et al.* (1973) *Virology* 52:456, Sambrook *et al.* (1989) *Molecular Cloning: a Laboratory Manual*, Cold Spring Harbor Laboratories, New York, Davis *et al.* (1986) *Basic Methods in Molecular Biology*, Elsevier, and Chu *et al.* (1981) *Gene* 13:197.

15 More particularly, there are two major steps in transfection: first, the exogenous DNA must traverse the recipient (host) cell plasma membrane in order to be exposed to the cell's transcription and replication machinery; and second, the DNA must either become stably integrated into the host cell genome, or be capable of extra-chromosomal replication at a sufficient rate. A number of transfection methods have been described

20 in the art, such as calcium phosphate co-precipitation (Graham *et al.* (1973) *Virol.* 52:456-467), direct micro-injection into cultured cells (Capecci, M. R. (1980) *Cell* 22:479-488), electroporation (Shigekawa *et al.* (1988) *BioTechniques* 6:742-751), liposome mediated gene transfer (Mannino *et al.* (1988) *BioTechniques* 6:682-690), lipid-mediated transfection (Felgner *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:7413-7417), and nucleic acid delivery using high-velocity microprojectiles (Klein *et al.* (1987) *Nature* 327:70-73).

The invention also relates to isolated proteins or polypeptides such as those encoded by nucleic acids of the present invention. Isolated proteins can be purified

from a natural source or can be made recombinantly. Proteins or polypeptides referred to herein as "isolated" are proteins or polypeptides that exist in a state different from the state in which they exist in cells in which they are normally expressed in an organism, and include proteins or polypeptides obtained by methods described herein, similar 5 methods or other suitable methods, and also include essentially pure proteins or polypeptides, proteins or polypeptides produced by chemical synthesis or by combinations of biological and chemical methods, and recombinant proteins or polypeptides which are isolated. Thus, the term "isolated" as used herein, indicates that the polypeptide in question exists in a physical milieu distinct from that in which it 10 occurs in nature. Thus, "isolated" includes existing in membrane fragments and vesicles, membrane fractions, liposomes, lipid bilayers and other artificial membrane systems. An isolated Ferroportin1 may be substantially isolated with respect to the complex cellular milieu in which it naturally occurs, and may even be purified essentially to homogeneity, for example as determined by PAGE or column 15 chromatography (for example, HPLC), but may also have further cofactors or molecular stabilizers, such as detergents, added to the purified protein to enhance activity. In one embodiment, proteins or polypeptides are isolated to a state at least about 75% pure; more preferably at least about 85% pure, and still more preferably at least about 95% pure, as determined by Coomassie blue staining of proteins on SDS-polyacrylamide 20 gels. Proteins or polypeptides referred to herein as "recombinant" are proteins or polypeptides produced by the expression of recombinant nucleic acids.

"Polypeptide" as used herein indicates a molecular chain of amino acids and does not refer to a specific length of the product. Thus, peptides, oligopeptides and proteins are included within the definition of polypeptide. This term is also intended to 25 include polypeptide that have been subjected to post-expression modifications such as, for example, glycosylations, acetylations, phosphorylations and the like.

In a preferred embodiment, an isolated polypeptide comprising a Ferroportin1, a functional portion thereof, or a functional equivalent of the Ferroportin1, has at least one

function characteristic of a Ferroportin1, for example, transport activity, binding function (e.g., a domain which binds to a cofactor), or antigenic function (e.g., binding of antibodies that also bind to a naturally-occurring Ferroportin1, as that function is found in an antigenic determinant). Functional equivalents can have activities that are 5 quantitatively similar to, greater than, or less than, the reference protein. These proteins include, for example, naturally occurring Ferroportin1 proteins that can be purified from tissues in which they are produced (including polymorphic or allelic variants), variants (e.g., mutants) of those proteins and/or portions thereof. Such variants include mutants differing by the addition, deletion or substitution of one or more amino acid residues, or 10 modified polypeptides in which one or more residues are modified, and mutants comprising one or more modified residues. The portions of the invention also include isolated polypeptides encoded by a nucleic acid molecule, wherein said nucleic acid molecule hybridizes to a complement of any of SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5 under high stringency conditions. Portions or fragments of a Ferroportin1 can 15 range in size from ten amino acid residues to the entire amino acid sequence minus one amino acid. An isolated polypeptide comprising a functional portion of a Ferroportin1 can comprise at least 10 amino acid residues of a cytoplasmic or extracellular domain of a Ferroportin1.

The isolated proteins of the invention preferably include mammalian iron 20 transport proteins of the Ferroportin1 family of homologous proteins. In preferred embodiments, the extent of amino acid sequence identity between a polypeptide having one of the amino acid sequences SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6, and the respective functional equivalents of these polypeptides is at least about 80% or 88%. In other embodiments, the degree of amino acid sequence identity between a Ferroportin1 25 and its respective functional equivalent is at least about 91%, at least about 94%, or at least about 97%.

The polypeptides of the invention also include those Ferroportin1 proteins encoded by polynucleotides which are orthologous to those polynucleotides, the

sequences of which are described herein in whole or in part. Ferroportin1 proteins which are orthologs to those described herein by amino acid sequence, in whole or in part, are, for example, Ferroportin1 proteins of dog, rat, chimpanzee, monkey, rabbit, guinea pig, baboon and pig, and are also embodiments of the invention.

5 To determine the percent identity of two amino acid sequences or of two nucleic acid sequences, the sequences are aligned for optimal comparison. In the simplest concept of identity, two nucleic acid sequences or two amino acid sequences are compared after aligning them for the maximum number of matches at the same position, without the introduction of any gaps. In a somewhat more complex concept of identity,
10 the sequences are aligned and gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment, and non-homologous (dissimilar) sequences can be disregarded for comparison purposes. In a preferred embodiment, the length of a reference sequence aligned for comparison purposes is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably
15 at least 60%, and even more preferably at least 70%, 80%, or 90% of the length of the reference sequence. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The
20 percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

The invention also encompasses polypeptides having a lower degree of identity
25 but having sufficient similarity in terms of structure and chemical characteristics so as to perform one or more of the same functions performed by the polypeptides described herein by amino acid sequence. Similarity for a polypeptide is determined by amino acid substitutions, which can be conservative amino acid substitutions. For example,

the invention encompasses polypeptides with at least one conservative amino acid substitution. Conservative substitutions are those that replace a given amino acid residue in a polypeptide with another amino acid residue of like characteristics. Conservative substitutions are likely to be phenotypically silent. Typically seen as

5 conservative substitutions are the replacements, one for another, among the aliphatic amino acids Ala, Val, Leu, and Ile; interchange of the hydroxyl residues Ser and Thr, exchange of the acidic residues Asp and Glu, substitution between the amide residues Asn and Gln, exchange of the basic residues Lys and Arg and replacements among the aromatic residues Phe, Tyr and Trp. Guidance concerning which amino acid changes

10 are likely to be phenotypically silent is found in Bowie *et al.*, *Science* 247:1306-1310 (1990).

TABLE: Conservative Amino Acid Substitutions

Aromatic		Phenylalanine	
		Tryptophan	
		Tyrosine	
Hydrophobic		Leucine	
		Isoleucine	
		Valine	
Polar		Glutamine	
		Asparagine	
5 Basic		Arginine	
		Lysine	
		Histidine	
Acidic		Aspartic Acid	
		Glutamic Acid	
Small		Alanine	
		Serine	
		Threonine	
		Methionine	
		Glycine	

The comparison of sequences and determination of percent similarity between two sequences can be accomplished using a mathematical algorithm. (*Computational Molecular Biology*, Lesk, A.M.,ed., Oxford University Press, New York, 1988; *Biocomputing: Informatics and Genome Projects*, Smith, D.W., ed., Academic Press, New York, 1993; *Computer Analysis of Sequence Data, Part 1*, Griffin, A.M., and

Griffin, H.G., eds., Humana Press, New Jersey, 1994; *Sequence Analysis in Molecular Biology*, von Heinje, G., Academic Press, 1987; and *Sequence Analysis Primer*, Gribskov, M. and Devereaux, J., eds., M. Stockton Press, New York, 1991). In a preferred embodiment, the percent similarity between two amino acid sequences is 5 determined using the Needleman and Wunsch (*J. Mol. Biol.* (48):444-453 (1970)) algorithm which has been incorporated into the GAP program in the Wisconsin Package Version 10.0, Genetics Computer Group (GCG), Madison, WI (see <http://www.gcg.com>), using, for example a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet another preferred 10 embodiment, the percent similarity between two nucleotide sequences is determined using the GAP program in the Wisconsin Package (Devereux, J., *et al.*, *Nucleic Acids Res.* 12(1):387 (1984)) (available at <http://www.gcg.com>), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. In another embodiment, the percent similarity between two amino acid or nucleotide 15 sequences is determined using the algorithm of E. Meyers and W. Miller (*CABIOS*, 4:11-17 (1989)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

The nucleic acids and protein sequences of the present invention can further be used as a "query sequence" to perform a search against databases to, for example, 20 identify other family members or related sequences. Such searches can be performed using the BLASTN, BLASTP, BLASTX, TBLASTN, TBLASTX programs (version 2.0) or PSI-BLAST 2.1 programs based on Altschul, *et al.* (*J. Mol. Biol.* 215:403-10 (1990)). BLAST nucleotide searches can be performed with the BLASTN program, for example, with default parameters matrix = BIOSUM62, gap existence cost = 11, per 25 residue gap cost = 1, lambda ratio = 0.85, filtered, to obtain nucleotide sequences homologous to (with calculatably significant similarity to) the nucleic acid molecules of the invention. BLAST protein searches can be performed with the BLASTP program, for example, with default parameters scoring matrix = BIOSUM62, word size = 3, E

value = 10, gap costs = 11,1 and alignments = 50, to obtain amino acid sequences homologous to the proteins of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.*, (*Nucleic Acids Res.* 25(17):3389-3402 (1997)). When utilizing BLAST and gapped

5 BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See <http://www.ncbi.nlm.nih.gov/BLAST/>.

Within the invention are isolated nucleic acid molecules having at least 80%, 85%, 90%, 95% and 97% sequence similarity to a nucleic acid encoding a polypeptide comprising the amino acid sequence SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6.

10 Also within the invention are isolated nucleic acid molecules which hybridize under high stringency conditions to nucleic acid consisting of the coding regions of SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6.

The invention further relates to fusion proteins, comprising a Ferroportin1 or functional portion thereof (as described above) as a first moiety, linked to second moiety
15 or to multiple moieties not occurring in the Ferroportin1 as found in nature. Thus, a second moiety can be an amino acid, peptide or polypeptide. The second moiety can be in an N-terminal location, C-terminal location or internal to the fusion protein, or multiple heterologous moieties can be in multiple locations. In one embodiment, the fusion protein comprises a Ferroportin1 or portion thereof having iron transport function
20 as the first moiety, and a second moiety comprising a linker sequence and an affinity ligand. Fusion proteins can be produced by a variety of methods. For example, a fusion protein can be produced by the insertion of a *ferroportin1* gene or portion thereof into a suitable expression vector, such as Bluescript SK +/- (Stratagene), pGEX-4T-2 (Pharmacia), pET-24(+) (Novagen), or vectors of similar construction. The resulting
25 construct can be introduced into a suitable host cell for expression. Upon expression, fusion protein can be purified from cells by means of a suitable affinity matrix (See e.g., *Current Protocols in Molecular Biology*, Ausubel, F.M. *et al.*, eds., Vol. 2, pp. 16.4.1-16.7.8, containing supplements up through Supplement 49, 2000).

The invention also relates to enzymatically produced, synthetically produced, or recombinantly produced portions of a Ferroportin1 protein. Portions of a Ferroportin1 can be made which have full or partial function on their own, or which when mixed together (though fully, partially, or nonfunctional alone), spontaneously assemble with 5 one or more other polypeptides to reconstitute a functional protein having at least one function characteristic of a Ferroportin1.

Fragments of a Ferroportin1 can be produced by direct peptide synthesis, for example those using solid-phase techniques (Roberge, J.Y. *et al.*, *Science* 269:202-204 (1995); Merrifield, J., *J. Am. Chem. Soc.* 85:2149-2154 (1963)). Peptide or polypeptide 10 synthesis can be performed using manual techniques or by automation. Automated synthesis can be carried out using, for instance, an Applied Biosystems 431A Peptide Synthesizer (Perkin Elmer). Various fragments of a Ferroportin1 can be synthesized separately and combined using chemical methods.

One aspect of the invention is a peptide or polypeptide having the amino acid 15 sequence of a portion of a Ferroportin1 protein which is hydrophilic rather than hydrophobic, and ordinarily can be detected as facing the outside of the cell membrane. Such a peptide or polypeptide can be thought of as being an extracellular domain of the Ferroportin1, or a mimetic of said extracellular domain. Peptides or polypeptides comprising at least 10 amino acid residues of a cytoplasmic or extracellular domain of 20 human, mouse or zebrafish Ferroportin1 can be synthesized.

The term "mimetic" as used herein, refers to a molecule, the structure of which is developed from knowledge of the structure of the Ferroportin1 of interest, or one or more portions thereof, and, as such, is able to effect some or all of the functions of a Ferroportin1.

25 Portions of a Ferroportin1 can be prepared by enzymatic cleavage of the isolated protein, or can be made by chemical synthesis methods. Portions of a Ferroportin1 can also be made by recombinant DNA methods in which restriction fragments, or fragments that may have undergone further enzymatic processing, or synthetically made

DNAs are joined together to construct an altered *ferroportin1* gene. The gene can be made such that it encodes one or more desired portions of a Ferroportin1. These portions of Ferroportin1 can be entirely homologous to a known Ferroportin1, or can be altered in amino acid sequence relative to naturally occurring Ferroportin1 proteins to 5 enhance or introduce desired properties such as solubility, stability, or affinity to a ligand. A further feature of the gene can be a sequence encoding an N-terminal signal peptide directed to the plasma membrane.

Another aspect of the invention relates to a method of producing a Ferroportin1 protein, variants or portions thereof, and to expression systems and host cells containing 10 a vector appropriate for expression of a Ferroportin1 protein.

Cells that express a Ferroportin1, a variant or a portion thereof, or an ortholog of a Ferroportin1 described herein by amino acid sequence, can be made and maintained in culture, under conditions suitable for expression, to produce protein in the cells for cell-based assays, or to produce protein for isolation. These cells can be prokaryotic or 15 eukaryotic. Examples of prokaryotic cells that can be used for expression include *Escherichia coli*, *Salmonella typhimurium* and *Bacillus subtilis*. Examples of eukaryotic cells that can be used for expression include yeasts such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Pichia pastoris* and other lower eukaryotic cells, and cells of higher eukaryotes such as those from insects and mammals, such as primary cells and 20 cell lines such as CHO, HeLa, 3T3, BHK, COS, human kidney 293 and Jurkat cells.

(See, e.g., Ausubel, F.M. *et al.*, eds. *Current Protocols in Molecular Biology*, Greene Publishing Associates and John Wiley & Sons, Inc., containing Supplements up through Supplement 49, 2000)).

In one embodiment, host cells that produce a recombinant Ferroportin1, or a 25 portion thereof, a variant, or an ortholog of a Ferroportin1 described herein by amino acid sequence, can be made as follows. A gene encoding a Ferroportin1, variant or a portion thereof can be inserted into a nucleic acid vector, e.g., a DNA vector, such as a plasmid, phage, cosmid, phagemid, virus, virus-derived vector (e.g., SV40, vaccinia,

adenovirus, fowl pox virus, pseudorabies viruses, retroviruses) or other suitable replicon, which can be present in a single copy or multiple copies, or the gene can be integrated in a host cell chromosome. A suitable replicon or integrated gene can contain all or part of the coding sequence for a Ferroportin1 or variant, operably linked to one or 5 more expression control regions whereby the coding sequence is under the control of transcription signals and linked to appropriate translation signals to permit translation. The vector can be introduced into cells by a method appropriate to the type of host cells (e.g., transfection, electroporation, infection). For expression from the Ferroportin1 gene, the host cells can be maintained under appropriate conditions (e.g., in the presence 10 of inducer, normal growth conditions, etc.). Proteins or polypeptides thus produced can be recovered (e.g., from the cells, as in a membrane fraction, from the periplasmic space of bacteria, from culture medium) using suitable techniques. Appropriate membrane targeting signal peptides may be incorporated into the expressed polypeptide. These signals may be endogenous to the polypeptide or they may be heterologous signal 15 peptides that do not naturally occur with a Ferroportin1.

Polypeptides of the invention can be recovered and purified from cell cultures (or from their primary cell source) by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity 20 chromatography, hydroxylapatite chromatography and high performance liquid chromatography. Known methods for refolding protein can be used to regenerate active conformation if the polypeptide is denatured during isolation or purification.

The host cells of the invention can be used to produce nonhuman transgenic animals. For example, in one embodiment, a host cell of the invention is a fertilized 25 oocyte or an embryonic stem cell into which Ferroportin1 coding sequences have been introduced. Such host cells can then be used to create non-human transgenic animals in which exogenous *ferroportin1* sequences have been introduced into their genome, or homologous recombinant animals in which endogenous *ferroportin1* sequences have

been altered. Such animals are useful for studying the function and/or activity of Ferroportin1, and for identifying and/or evaluating modulators of Ferroportin1 activity. As used herein, a “transgenic animal” is a non-human animal, preferably a mammal, more preferably a rodent such as a rat or mouse, in which one or more of the cells of the 5 animal includes a transgene. Other examples of transgenic animals include non-human primates, sheep, dogs, cows, goats, chickens, amphibians, etc. A transgene is exogenous DNA which is integrated into the genome of a cell from which a transgenic animal develops and which remains in the genome of the mature animal, thereby directing the expression of an encoded gene product in one or more cell types or tissues 10 of the transgenic animal. “Exogenous” as used in the context of a transgenic animal, means different from that of the unaltered recipient host cell. As used herein, a “homologous recombinant animal” is a non-human animal, preferably a mammal, more preferably a mouse, in which an endogenous *weh* gene has been altered by homologous recombination between the endogenous gene and an exogenous DNA molecule 15 introduced into a cell of the animal, e.g., an embryonic cell of the animal, prior to development of the animal.

A transgenic animal of the invention can be created by introducing Ferroportin1 encoding nucleic acid into the male pronuclei of a fertilized oocyte, e.g., by microinjection, retroviral infection, and allowing the oocyte to develop in a 20 pseudopregnant female foster animal. The *ferroportin1* cDNA sequence can be introduced as a transgene into the genome of a non-human animal. Alternatively, a nonhuman homolog of the human *ferroportin1* gene can be isolated based on hybridization to the human or mouse *ferroportin1* cDNA and used as a transgene. 25 Intronic sequences and polyadenylation signals can also be included in the transgene to increase the efficiency of expression of the transgene. One or more tissue-specific regulatory sequences can be operably linked to the *ferroportin1* transgene to direct expression of Ferroportin1 protein to particular cells. Methods for generating transgenic animals via embryo manipulation and microinjection, particularly animals such as mice,

1242.1035-002

have become conventional in the art and are described, for example, in U.S. Pat. Nos. 4,736,866 and 4,870,009, U.S. Pat. No. 4,873,191 and in Hogan, *Manipulating the Mouse Embryo* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986). Similar methods are used for production of other transgenic animals. A

5 transgenic founder animal can be identified based upon the presence of the *ferroportin1* transgene in its genome and/or expression of *ferroportin1* mRNA in tissues or cells of the animals. A transgenic founder animal can then be used to breed additional animals carrying the transgene. Transgenic animals carrying a transgene encoding Ferroportin1 can further be bred to other transgenic animals carrying other transgenes.

10 To create a homologous recombinant animal, a vector is prepared which contains at least a portion of a *ferroportin1* gene (e.g., a human or a non-human homolog of the gene encoding Ferroportin1, e.g., a murine *ferroportin1* gene) into which a deletion, addition or substitution has been introduced to thereby alter, e.g., functionally disrupt, the *ferroportin1* gene. In a preferred embodiment, the vector is 15 designed such that, upon homologous recombination, the endogenous *ferroportin1* gene is functionally disrupted (i.e., no longer encodes a functional protein; also referred to as a “knock out” vector).

Alternatively, the vector can be designed such that, upon homologous recombination, the endogenous *ferroportin1* gene is mutated or otherwise altered but 20 still encodes functional protein (e.g., the upstream regulatory region can be altered to thereby alter the expression of the endogenous Ferroportin1 protein). In the homologous recombination vector, the altered portion of the *ferroportin1* gene is flanked at its 5' and 3' ends by additional nucleic acid of the *ferroportin1* gene to allow 25 for homologous recombination to occur between the exogenous *ferroportin1* gene carried by the vector and an endogenous *ferroportin1* gene in an embryonic stem cell. The additional flanking *ferroportin1* nucleic acid is of sufficient length for successful homologous recombination with the endogenous gene. Typically, several kilobases of flanking DNA (both at the 5' and 3' ends) are included in the vector (see, e.g., Thomas

and Capecchi (1987) *Cell* 51:503 for a description of homologous recombination vectors). The vector is introduced into an embryonic stem cell line (e.g., by electroporation) and cells in which the introduced *ferroportin1* gene has homologously recombined with the endogenous *ferroportin1* gene are selected (see., e.g., Li *et al.* 5 (1992) *Cell* 69:915). The selected cells are then injected into a blastocyst of an animal (e.g., a mouse) to form aggregation chimeras (see, e.g., Bradley in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, Robertson, ed. (IRL, Oxford, 1987) pp. 113-152). A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term. Progeny harboring the 10 homologously recombined DNA in their germ cells can be used to breed animals in which all cells of the animal contain the homologously recombined DNA by germline transmission of the transgene. Methods for constructing homologous recombination vectors and homologous recombinant animals are described further in Bradley (1991) *Current Opinion in Bio/Technology* 2:823-829 and in WO 90/11354, WO 91/01140, 15 WO 92/0968, and WO 93/04169.

In another embodiment, transgenic non-human animals can be produced which contain selected systems which allow for regulated expression of the transgene. One example of such a system is the *cre/loxP* recombinase system of bacteriophage P1. For a description of the *cre/loxP* recombinase system, see, e.g., Lakso *et al.* (1992) *Proc. 20 Natl. Acad. Sci. USA* 89:6232-6236. Another example of a recombinase system is the FLP recombinase system of *Saccharomyces cerevisiae* (O'Gorman *et al.* (1991) *Science* 251:1351-1355). If a *cre/loxP* recombinase system is used to regulate expression of the transgene, animals containing transgenes encoding both the Cre recombinase and a selected protein are required. Such animals can be provided through the construction of 25 "double" transgenic animals, e.g., by mating two transgenic animals, one containing a transgene encoding a selected protein and the other containing a transgene encoding a recombinase.

Clones of the non-human transgenic animals described herein can also be produced according to the methods described in Wilmut *et al.* (1997) *Nature* 385:810-813 and WO 97/07668 and WO 97/07669. In brief, a cell, e.g., a somatic cell, from the transgenic animal can be isolated and induced to exit the growth cycle and enter G₀ phase. The quiescent cell can then be fused, e.g., through the use of electrical pulses, to an enucleated oocyte from an animal of the same species from which the quiescent cell is isolated. The reconstructed oocyte is then cultured such that it develops to morula or blastocyst and then transferred to pseudopregnant female foster animal. The offspring borne of this female foster animal will be a clone of the animal from which the cell, e.g., the somatic cell, is isolated.

In a further aspect of the invention are methods for assessing the transport function of any of the Ferroportin1 proteins or polypeptides described herein, including orthologs, and in variations of these, methods for identifying an inhibitor (or an enhancer) of such function and methods for assessing the transport function in the presence of a candidate inhibitor or a known inhibitor.

A variety of systems comprising living cells can be used for these methods. Cells to be used in iron transport assays, and further in methods for identifying an inhibitor or enhancer of this function, express one or more Ferroportin1 proteins. Cells for use in cell-based assays described herein can be drawn from a variety of sources, such as isolated primary cells of various organs and tissues wherein a Ferroportin1 protein is naturally expressed. In some cases, the cells can be from adult organs, and in some cases, from embryonic or fetal structures, such as placenta, yolk sac, heart, lung, liver, intestine, skeletal muscle, kidney and the like. Cells for this purpose can also include cells cultured as fragments of organs or in conditions simulating the cell type and/or tissue organization of organs, in which artificial materials may be used as substrates for cell growth. Other types of cells suitable for this purpose include cells of a cell strain or cell line (ordinarily comprising cells considered to be "transformed") transfected to express one or more types of Ferroportin1.

A further embodiment of the invention is a method for detecting, in a sample of cells, a Ferroportin1 protein, a portion or fragment thereof, a fusion protein comprising a Ferroportin1 or a portion thereof, or an ortholog as described herein, wherein the cells can be, for instance, cells of a tissue, primary culture cells, or cells of a cell line,

5 including cells into which nucleic acid has been introduced. The method comprises adding to the sample an agent that specifically binds to the protein, and detecting the agent specifically bound to the protein. Appropriate washing steps can be added to reduce nonspecific binding to the agent. The agent can be, for example, an antibody, a ligand or a substrate or cofactor mimic. The agent can have incorporated into it, or have

10 bound to it, covalently or by high affinity non-covalent interactions, for instance, a label that facilitates detection of the agent to which it is bound, wherein the label can be, but is not limited to, a phosphorescent label, a fluorescent label, a biotin or avidin label, or a radioactive label. The means of detection of a Ferroportin1 can vary, as appropriate to the agent and label used. For example, for an antibody that binds to the Ferroportin1,

15 the means of detection may call for binding a second antibody, which has been conjugated to an enzyme, to the antibody which binds the Ferroportin1, and detecting the presence of the second antibody by means of the enzymatic activity of the conjugated enzyme.

Similar principles can also be applied to a cell lysate, membrane fraction, or a

20 more purified preparation of proteins from cells that may comprise a Ferroportin1 protein of interest, for example in the methods of immunoprecipitation, immunoblotting, immunoaffinity methods, that in addition to detection of the particular Ferroportin1, can also be used in purification steps, and qualitative and quantitative immunoassays. See, for instance, chapters 11 through 14 in *Antibodies: A Laboratory*

25 *Manual*, E. Harlow and D. Lane, eds., Cold Spring Harbor Laboratory, 1988.

Isolated Ferroportin1 protein or, an antigenically similar portion thereof, especially a portion that is soluble (e.g., a peptide or a fusion polypeptide comprising at least 10 contiguous amino acid residues of a Ferroportin1), can be used in a method to

select and identify molecules which bind specifically to the Ferroportin1. Fusion proteins comprising all of, or a portion of, the Ferroportin1 linked to a second moiety not occurring in the Ferroportin1 as found in nature, can be prepared for use in another embodiment of the method. Suitable fusion proteins for this purpose include those in

5 which the second moiety comprises an affinity ligand (e.g., an enzyme, antigen, epitope). Ferroportin1 fusion proteins can be produced by the insertion of a gene encoding the Ferroportin1 or a variant thereof, or a suitable portion of such gene into a suitable expression vector which encodes an affinity ligand (e.g., pGEX-4T-2 and pET-15b, encoding glutathione S-transferase and His-Tag affinity ligands, respectively). The

10 expression vector can be introduced into a suitable host cell for expression. Host cells are lysed and the lysate, containing fusion protein, can be bound to a suitable affinity matrix by contacting the lysate with an affinity matrix.

In one embodiment, the fusion protein can be immobilized on a suitable affinity matrix under conditions sufficient to bind the affinity ligand portion of the fusion protein to the matrix, and is contacted with one or more candidate binding agents (e.g., a mixture of peptides or compounds of a library) to be tested, under conditions suitable for binding of the binding agents to the Ferroportin1 portion of the bound fusion protein. Next, the affinity matrix with bound fusion protein can be washed with a suitable wash buffer to remove unbound candidate binding agents and non-specifically bound candidate binding agents. Those agents which remain bound can be released by contacting the affinity matrix with fusion protein bound thereto with a suitable elution buffer. Wash buffer can be formulated to permit binding of the fusion protein to the affinity matrix, without significantly disrupting binding of specifically bound binding agents. In this aspect, elution buffer can be formulated to permit retention of the fusion protein by the affinity matrix, but can be formulated to interfere with binding of the candidate binding agents to the target portion of the fusion protein. For example, a change in the ionic strength or pH of the elution buffer can lead to release of specifically bound agent, or the elution buffer can comprise a release component or components

designed to disrupt binding of specifically bound agent to the target portion of the fusion protein.

Immobilization can be performed prior to, simultaneous with, or after, contacting the fusion protein with candidate binding agent, as appropriate. Various 5 permutations of the method are possible, depending upon factors such as the candidate molecules tested, the affinity matrix-ligand pair selected, and elution buffer formulation. For example, after the wash step, fusion protein with binding agent molecules bound thereto can be eluted from the affinity matrix with a suitable elution buffer (a matrix elution buffer, such as glutathione for a GST fusion). Where the fusion protein 10 comprises a cleavable linker, such as a thrombin cleavage site, cleavage from the affinity ligand can release a portion of the fusion with the candidate agent bound thereto. Bound agent molecules can then be released from the fusion protein or its cleavage product by an appropriate method, such as extraction.

One or more candidate binding agents can be tested simultaneously. Where a 15 mixture of candidate binding agents is tested, those found to bind by the foregoing processes can be separated (as appropriate) and identified by suitable methods (e.g., PCR, sequencing, chromatography). Large libraries of candidate binding agents produced by combinatorial chemical synthesis or by other methods can be tested (see e.g., Ohlmeyer, M.H.J. *et al.*, *Proc. Natl. Acad. Sci. USA* 90:10922-10926 (1993) and 20 DeWitt, S.H. *et al.*, *Proc. Natl. Acad. Sci. USA* 90:6909-6913 (1993), relating to tagged compounds; see also Rutter, W.J. *et al.* U.S. Patent No. 5,010,175; Huebner, V.D. *et al.*, U.S. Patent No. 5,182,366; and Geysen, H.M., U.S. Patent No. 4,833,092). Where binding agents selected from a combinatorial library by the present method carry unique 25 tags, identification of individual biomolecules by chromatographic methods is possible. Where binding agents do not carry tags, chromatographic separation, followed by mass spectrometry to ascertain structure, can be used to identify binding agents selected by the method, for example.

The invention also comprises a method for identifying an agent which inhibits interaction between a Ferroportin1 protein (e.g., one comprising the amino acid sequence in SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6), and a ligand of said protein. The Ferroportin1 can be one described by amino acid sequence herein, a portion or fragment thereof, a variant thereof, or an ortholog thereof, or a Ferroportin1 fusion protein. Here, a ligand can be, for instance, a substrate (e.g., Fe^{2+}), or a substrate mimic, an antibody, or a compound, such as a small molecule or peptide, that binds with specificity to a site on the protein. The method comprises combining, not limited to a particular order, the Ferroportin1 protein, the ligand of the protein, and a candidate agent to be assessed for its ability to inhibit interaction between the protein and the ligand, under conditions appropriate for interaction between the protein and the ligand (e.g., pH, salt, temperature conditions conducive to appropriate conformation and molecular interactions); determining the extent to which the protein and ligand interact; and comparing (1) the extent of protein-ligand interaction in the presence of candidate agent with (2) the extent of protein-ligand interaction in the absence of candidate agent, wherein if (1) is less than (2), then the candidate agent is one which inhibits interaction between the protein and the ligand.

The method can be facilitated, for example, by using an experimental system which employs a solid support (column chromatography matrix, wall of a plate, 20 microtiter wells, column pore glass, pins to be submerged in a solution, beads, etc.) to which the protein can be attached. Accordingly, in one embodiment, the protein can be fixed to a solid phase directly or indirectly, by a linker. The candidate agent to be tested is added under conditions conducive for interaction and binding to the protein. The ligand is added to the solid phase system under conditions appropriate for binding. 25 Excess ligand is removed, as by a series of washes done under conditions that do not disrupt protein-ligand interactions. Detection of bound ligand can be facilitated by using a ligand that carries a label (e.g., fluorescent, chemiluminescent, radioactive). In a control experiment, protein and ligand are allowed to interact in the absence of any

candidate agent, under conditions otherwise identical to those used for the "test" conditions where candidate inhibiting agent is present, and any washes used in the test conditions are also used in the control. The extent to which ligand binds to the protein in the presence of candidate agent is compared to the extent to which ligand binds to the 5 protein in the absence of the candidate agent. If the extent to which interaction of the protein and the ligand occurs is less in the presence of the candidate agent than in the absence of the candidate agent, the candidate agent is an agent which inhibits interaction between the protein and the ligand of the protein.

In a further embodiment, an inhibitor (or an enhancer) of a Ferroportin1 protein 10 can be identified. The method comprises steps which are, or are variations of, the following: contacting the cells with Fe^{2+} under conditions allowing uptake of the Fe^{2+} , wherein the Fe^{2+} can be labeled for convenience of detection; washing away extracellular Fe^{2+} , contacting a first aliquot of the cells with an agent being tested as an 15 inhibitor (or enhancer) of iron export, while maintaining a second aliquot of cells under the same conditions but without contact with the agent; and determining (e.g., by a quantitative measurement) iron exported from the first and second aliquots of cells; wherein a lesser quantity of iron in the first aliquot compared to that in the second aliquot is indicative that the agent is an inhibitor of iron export by a Ferroportin1 protein. A greater quantity of extracellular iron found in the first aliquot compared to 20 that in the second aliquot is indicative that the agent is an enhancer of iron export by a Ferroportin1 protein.

A particular embodiment of identifying an inhibitor or enhancer of iron export function employs the above steps, but also employs additional steps preceding those given above: introducing into cells of a cell strain or cell line ("host cells" for the 25 intended introduction of, or after the introduction of, a vector) one or more vectors or RNAs comprising a *ferroportin1* gene, wherein expression of the gene can be regulatable or constitutive, and providing conditions to the host cells under which

expression of the gene can occur, and under which iron can be taken up by the host cells.

The terms "contacting" and "combining" as used herein in the context of bringing molecules into close proximity to each other, can be accomplished by 5 conventional means. For example, when referring to molecules that are soluble, contacting is achieved by adding the molecules together in a solution. "Contacting" can also be adding an agent to a test system, such as a vessel containing cells in tissue culture.

The term "inhibitor" or "antagonist", as used herein, refers to an agent which 10 blocks, diminishes, inhibits, hinders, limits, decreases, reduces, restricts or interferes with iron export from a cell, or alternatively or additionally, prevents or impedes the cellular effects associated with iron export. The term "enhancer" or "agonist", as used herein, refers to an agent which augments, enhances, or increases iron export from a cell.

15 In order to produce a "host cell" type suitable for iron uptake assays and for assays derived therefrom for identifying inhibitors or enhancers thereof, a nucleic acid vector can be constructed to comprise a gene encoding an iron transport protein, for example, human Ferroportin1, a mutant or variant thereof, an ortholog of the human protein, such as porcine or bovine orthologs or orthologs found in other mammals, or a 20 Ferroportin1 family protein of origin in an organism other than a mammal. The gene of the vector can be regulatable, such as by the placement of the gene under the control of an inducible or repressible promoter in the vector (e.g., inducible or repressible by a change in growth conditions of the host cell harboring the vector, such as addition of inducer, binding or functional removal of repressor from the cell milieu, or change in 25 temperature) such that expression of the *ferroportin1* gene can be turned on or initiated by causing a change in growth conditions, thereby causing the protein encoded by the gene to be produced, in host cells comprising the vector, as a plasma membrane protein. Alternatively, the *ferroportin1* gene can be constitutively expressed.

A vector comprising a *ferroportin1* gene, such as a vector described herein, can be introduced into host cells by a means appropriate to the vector and to the host cell type. For example, commonly used methods such as electroporation, transfection, for instance, transfection using CaCl₂, and transduction (as for a virus or bacteriophage) can 5 be used. Host cells can be, for example, mammalian cells such as primary culture cells or cells of cell lines such as COS cells, 293 cells or Jurkat cells. Host cells can also be, in some cases, cells derived from insects, cells of insect cell lines, bacterial cells, such as *E. coli*, or yeast cells, such as *S. cerevisiae*. It is preferred that the iron export protein whose function is to be assessed, with or without a candidate inhibitor or 10 enhancer, be produced in host cells whose ancestor cells originated in a species related to the species of origin of the *ferroportin1* gene encoding the Ferroportin1 protein. For example, it is preferable that tests of function or of inhibition or enhancement of a mammalian Ferroportin1 be carried out in host mammalian cells producing the Ferroportin1, rather than in bacterial cells or yeast cells.

15 Host cells comprising a vector comprising a regulatable *ferroportin1* gene can be treated so as to allow expression of the *ferroportin1* gene and production of the encoded protein (e.g., by contacting the cells with an inducer compound that effects transcription from an inducible promoter operably linked to the *ferroportin1* gene).

The test agent (e.g., an agonist or antagonist) is added to the cells to be used in 20 an iron export assay, under conditions suitable for production and/or maintenance of the expressed Ferroportin1 in a conformation appropriate for association of the Ferroportin1 with test agent and substrate. For example, conditions under which an agent is assessed, such as media and temperature requirements, can initially be similar to those necessary for transport of iron substrate across the plasma membrane. One of ordinary skill in the 25 art will know how to vary experimental conditions depending upon the biochemical nature of the test agent. The test agent can be added to the cells before or after the addition of an iron substrate. The concentration at which the test agent can be evaluated

can be varied, as appropriate, to test for an increased effect with increasing concentrations.

Test agents to be assessed for their effects on iron transport can be any chemical (element, molecule, compound), made synthetically, made by recombinant techniques or 5 isolated from a natural source. For example, test agents can be peptides, polypeptides, peptoids, sugars, hormones, or nucleic acid molecules, such as antisense nucleic acid molecules. In addition, test agents can be small molecules or molecules of greater complexity made by combinatorial chemistry, for example, and compiled into libraries. These libraries can comprise, for example, alcohols, alkyl halides, amines, amides, 10 esters, aldehydes, ethers and other classes of organic compounds. Test agents can also be natural or genetically engineered products isolated from lysates of cells, bacterial, animal or plant, or can be the cell lysates themselves. Presentation of test compounds to the test system can be in either an isolated form or as mixtures of compounds, especially in initial screening steps.

15 Thus, the invention relates to a method for identifying agents which alter iron export, the method comprising providing the test agent to the cell (wherein "cell" includes the plural, and can include cells of a cell strain, cell line or culture of primary cells or organ culture, for example), under conditions suitable for binding to its target, whether to the Ferroportin1 itself or to another target on or in the cell, wherein the cell 20 comprises a Ferroportin1.

The cells to be tested for the effect of an agent on iron export can be "loaded" with iron by incubation of the cells with iron under conditions appropriate for iron uptake. The cells can be, for example, cells of transformed cell lines such as HeLa or 293 cells, fibroblasts, transformed fibroblasts or oocytes of *Xenopus laevis* or another 25 appropriate species. The cells can also be cells transfected with nucleic acid encoding Ferroportin1, such that the cell expresses the Ferroportin1 protein to be tested for the effect of an agent. The iron can be labeled to facilitate its detection, for example, with a radioactive isotope.

The cells so loaded with iron are then washed with buffer or medium sufficient to remove iron external to the cells. The cells can then be divided into two equal aliquots, or two aliquots of known cell numbers. To one aliquot is added the agent to be tested for its effect on iron transport. To the other aliquot is added a volume of buffer, 5 medium, etc. equivalent to that in which the agent added to the first aliquot was dissolved. The two aliquots of cells are then kept under the same culture conditions for a period of time to allow for the export of iron. After this period, the cells of each aliquot are separated from their surrounding medium, for example by centrifugation, and, for isolation of the cell pellet, by one or more additional washing steps. The 10 medium can be collected in each case, and aliquots of each can be assayed for exported iron. Where the iron is radioactively labeled, the medium can be tested for radioactivity, as by scintillation counting. Alternatively, the cells or aliquots of the cells can be collected after the period of time allowing for iron export, and the cells can be lysed to prepare a cell extract to be assayed for iron retained in the cells. Where the 15 cells to which an agent was added retain more iron than the control cells not receiving agent, the agent is an inhibitor of iron export. Where the cells to which an agent was added retain less iron than the control cells, the agent is an enhancer of iron export. If the cell medium is assayed, where the cells receiving agent export less iron into the medium than the control cells, then the agent is an inhibitor of iron transport. If the 20 cells receiving agent export more iron into the medium than the control cells, then the agent is an enhancer of iron transport.

An agent determined to be an inhibitor (or enhancer) of Ferroportin1 function, such as iron binding and/or iron export, can be administered to cells in culture, or *in vivo*, to a mammal (e.g. human) to inhibit (or enhance) Ferroportin1 function. Such an 25 agent may be one that acts directly on the Ferroportin1 protein (for example, by binding) or can act on an intermediate in a biosynthetic pathway to produce Ferroportin1, such as transcription of the *ferroportin1* gene, processing of the mRNA, or translation of the mRNA. An example of such an agent is antisense oligonucleotide.

Cell-free assays can also be used to measure the transport of iron across a membrane, and therefore also to assess a test treatment or test agent for its effect on the rate or extent of iron transport. Isolated Ferroportin1, for example in the presence of a detergent that preserves the native 3-dimensional structure of the Ferroportin1 protein, 5 or partially purified Ferroportin1 protein, can be used in an artificial membrane system typically used to preserve the native conformation and activity of membrane proteins. Such systems include liposomes, artificial bilayers of phospholipids, isolated plasma membrane such as cell membrane fragments, cell membrane fractions, or cell membrane vesicles, and other systems in which the Ferroportin1 protein can be properly oriented 10 within the membrane to have transport activity. Assays for transport activity can be performed using methods analogous to those that can be used in cells expressing a Ferroportin1 protein whose function is to be measured. A labeled (e.g., radioactively labeled) iron substrate can be incubated on one side of a bilayer or in a suspension of liposomes constructed to integrate a properly oriented Ferroportin1 protein. The 15 accumulation of iron with time can be measured, using appropriate means to detect the label (e.g., scintillation counting of medium on each side of the bilayer, or of the contents of liposomes versus the surrounding medium). Assays such as these can be adapted to use for the testing of agents which might interact with the Ferroportin1 to produce an inhibitory or an enhancing effect on the rate or extent of iron transport. That 20 is, the above-described assay can be done in the presence or absence of the agent to be tested, and the results compared.

For examples of isolation of membrane proteins (ADP/ATP carrier and uncoupling protein), reconstitution into phospholipid vesicles, and assays of transport, see Klingenberg, M. *et al.*, *Methods Enzymol.* 260:369-389 (1995). For an example of a 25 membrane protein (phosphate carrier of *Saccharomyces cerevisiae*) that was purified and solubilized from *E. coli* inclusion bodies, see Schroer, A. *et al.*, *J. Biol. Chem.* 273:14269-14276 (1998). The Glut1 glucose transporter of rat has been expressed in yeast. A crude membrane fraction of the yeast was prepared and reconstituted with

soybean phospholipids into liposomes. Glucose transport activity could be measured in the liposomes (Kasahara, T. and Kasahara, M., *J. Biol. Chem.* 273:29113-29117 (1998)). Similar methods can be applied to the proteins and polypeptides of the invention.

5 Another embodiment of the invention is a method for inhibiting iron export in Ferroportin1-expressing cells of a mammal (e.g., a human), comprising administering to the mammal a therapeutically effective amount of an inhibitor of the transport function of Ferroportin1, thereby decreasing iron in the circulation. Hemochromatosis can be due to the inheritance of a mutant gene or due to secondary iron overload from an iron-
10 loading anemia such as thalassemia or sideroblastic anemia. Where it is desirable to reduce the uptake of iron into the circulatory system through the intestine, for example, in the treatment of hemochromatosis in a human, one or more inhibitors of Ferroportin1 can be administered in an effective dose, and by an effective route, for example, orally, or by an indwelling device that can deliver doses to the small intestine. The inhibitor
15 can be one identified by methods described herein, or can be one that is, for instance, structurally related to an inhibitor identified by methods described herein (e.g., having chemical adducts to better stabilize or solubilize the inhibitor). The invention further relates to compositions comprising inhibitors of iron uptake in a mammal, which may further comprise pharmaceutical carriers suitable for administration to a subject
20 mammal, such as sterile solubilizing or emulsifying agents.

A further embodiment of the present invention is a method of enhancing or increasing iron uptake into the body, such as enhancing or increasing iron uptake in the small intestine (e.g., to treat a malabsorption syndrome or anemia). In this embodiment, a therapeutically effective amount of an enhancer of the transport function of
25 Ferroportin1 can be administered to a mammalian subject, with the result that iron uptake in the small intestine is enhanced. In this embodiment, one or more enhancers of a Ferroportin1 protein is administered in an effective dose and by a route (e.g., orally or by a device, such as an indwelling catheter or other device) which can deliver doses to

the gut. The enhancer of Ferroportin1 function can be identified by methods described herein or can be one that is structurally similar to an enhancer identified by methods described herein.

The invention further relates to antibodies that bind to an isolated or 5 recombinant Ferroportin1, including portions of antibodies, which can specifically recognize and bind to one or more Ferroportin1 proteins. The antibodies and portions thereof of the invention include those which bind to Ferroportin1 proteins of zebrafish, or Ferroportin1 proteins of mouse or other mammalian species. In a specific embodiment, the antibodies bind to a naturally occurring Ferroportin1 of humans. The 10 antibodies can be used in various methods to detect or to purify a protein of the present invention or a portion thereof such as ELISA, western blotting or immunoaffinity chromatography, to inhibit the function of a protein in a method of therapy, or to selectively inactivate an active site, or to study other aspects of the structure of these proteins, for example.

15 The antibodies of the present invention can be polyclonal or monoclonal. The term antibody is intended to encompass both polyclonal and monoclonal antibodies. Antibodies of the present invention can be raised against an appropriate immunogen, including proteins or polypeptides of the present invention, such as an isolated or recombinant Ferroportin1 or portions thereof, or synthetic molecules, such as synthetic 20 peptides (e.g., conjugated to a suitable carrier). Preferred embodiments are antibodies that bind to any of the following, and which may cross-react with Ferroportin1 proteins of several species: zebrafish Ferroportin1, mouse Ferroportin1, or human Ferroportin1. The immunogen can be a polypeptide comprising a portion of a Ferroportin1 and having at least one function of a Ferroportin1, as described herein. To produce polyclonal 25 antibodies, the immunogen is introduced into an animal that is not the original source of the immunogen (e.g., mouse Ferroportin1 or a fragment thereof injected into a non-murine animal).

The term antibody is also intended to encompass single chain antibodies, chimeric, humanized or primatized (CDR-grafted) antibodies and the like, as well as chimeric or CDR-grafted single chain antibodies, comprising portions from more than one species. For example, the chimeric antibodies can comprise portions of proteins

5 derived from two different species, joined together chemically by conventional techniques or prepared as a single contiguous protein using genetic engineering techniques (e.g., DNA encoding the protein portions of the chimeric antibody can be expressed to produce a contiguous protein chain. See, e.g., Cabilly *et al.*, U.S. Patent No. 4,816,567; Cabilly *et al.*, European Patent No. 0,125,023 B1; Boss *et al.*, U.S.

10 Patent No. 4,816,397; Boss *et al.*, European Patent No. 0,120,694 B1; Neuberger, M.S. *et al.*, WO 86/01533; Neuberger, M.S. *et al.*, European Patent No. 0,194,276 B1; Winter, U.S. Patent No. 5,225,539; Winter, European Patent No. 0,239,400 B1; Queen *et al.*, U.S. Patent No. 5,585,089; and Queen *et al.*, European Patent No. EP 0 451 216 B1. See also, Newman, R. *et al.*, *BioTechnology*, 10:1455-1460 (1992), regarding

15 primatized antibody, and Ladner *et al.*, U.S. Patent No. 4,946,778 and Bird, R.E. *et al.*, *Science*, 242:423-426 (1988) regarding single chain antibodies.)

Whole antibodies and biologically functional fragments thereof are also encompassed by the term antibody. Biologically functional antibody fragments which can be used include those fragments sufficient for binding of the antibody fragment to a

20 Ferroportin1 to occur, such as Fv, Fab, Fab' and F(ab')₂ fragments. Such fragments can be produced by enzymatic cleavage or by recombinant techniques. For instance, papain or pepsin cleavage can generate Fab or F(ab')₂ fragments, respectively. Antibodies can also be produced in a variety of truncated forms using antibody genes in which one or more stop codons have been introduced upstream of the natural stop site. For example,

25 a chimeric gene encoding a F(ab')₂ heavy chain portion can be designed to include DNA sequences encoding the CH₁ domain and hinge region of the heavy chain.

Preparation of immunizing antigen (for instance, whole cells comprising a Ferroportin1 on the cell surface, or a purified Ferroportin1), and polyclonal and

monoclonal antibody production can be performed using any suitable technique. A variety of methods have been described for the production of antibodies (See e.g., Kohler *et al.*, *Nature*, 256:495-497 (1975) and *Eur. J. Immunol.* 6: 511-519 (1976); Milstein *et al.*, *Nature* 266:550-552 (1977); Koprowski *et al.*, U.S. Patent No. 5 4,172,124; Harlow, E. and D. Lane, 1988, *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory: Cold Spring Harbor, NY); Chapter 11 In *Current Protocols In Molecular Biology*, Vol. 2 (containing supplements up through Supplement 49, 2000), Ausubel, F.M. *et al.*, eds., John Wiley & Sons: New York, NY). Generally, a hybridoma can be produced by fusing a suitable immortal cell line (e.g., a myeloma cell line such as SP2/0) with antibody producing cells. The antibody producing cells, preferably those obtained from the spleen or lymph nodes, can be obtained from animals immunized with the antigen of interest. Immunization of animals can be, for instance, by introduction of whole cells comprising Ferroportin1 protein on the cell surface. The fused cells (hybridomas) can be isolated using selective culture conditions, and cloned by limiting dilution. Cells which produce antibodies with the desired specificity can be selected by a suitable assay (e.g., ELISA).

Other suitable methods of producing or isolating antibodies (including human antibodies) of the requisite specificity can be used, including, for example, methods which select recombinant antibody from a library (e.g., Hoogenboom *et al.*, WO 93/06213; 20 Hoogenboom *et al.*, U.S. Patent No. 5,565,332; WO 94/13804, published June 23, 1994; and Dower, W.J. *et al.*, U.S. Patent No. 5,427,908), or which rely upon immunization of transgenic animals (e.g., mice) capable of producing a full repertoire of human antibodies (see e.g., Jakobovits *et al.*, *Proc. Natl. Acad. Sci. USA*, 90: 2551-2555 (1993); Jakobovits *et al.*, *Nature*, 362:255-258 (1993); Lonberg *et al.*, U.S. Patent No. 25 5,569,825; Lonberg *et al.*, U.S. Patent No. 5,545,806; Surani *et al.*, U.S. Patent No. 5,545,807; and Kucherlapati, R. *et al.*, European Patent No. EP 0 463 151 B1).

The invention also relates to compositions comprising a modulator of Ferroportin1 function. The term "modulate" as used herein refers to the ability of a

molecule to alter the function of another molecule. Thus, modulate could mean, for example, inhibit, antagonize, agonize, upregulate, downregulate, induce, or suppress. A modulator has the capability of altering function of its target. Such alteration can be accomplished at any stage of the transcription, translation, expression or function of the 5 protein, so that, for example, modulation of a target gene can be accomplished by modulation of the DNA or RNA encoding the protein, and the protein itself.

Antagonists or agonists (inhibitors or enhancers) of the Ferroportin1 proteins of the invention, antibodies that bind a Ferroportin1, or mimetics of a Ferroportin1 or of portions of a Ferroportin1 can be employed in combination with a non-sterile or sterile 10 carrier or carriers for use with cells, tissues or organisms, such as a pharmaceutical carrier suitable for administration to a mammalian subject. Such compositions comprise, for instance, a media additive or a therapeutically effective amount of an inhibitor or enhancer compound to be identified by an assay of the invention and a pharmaceutically acceptable carrier or excipient. Such carriers may include, but are not 15 limited to, saline, buffered saline, dextrose, water, ethanol, surfactants, such as glycerol, excipients such as lactose and combinations thereof. The formulation can be chosen by one of ordinary skill in the art to suit the mode of administration. The chosen route of administration will be influenced by the predominant tissue or organ location of the Ferroportin1 wherein it is intended that function is to be inhibited or enhanced. For 20 example, for affecting the function of ferroportin1 in the duodenum, a particular administration can be oral or through a tube inserted into the stomach (e.g., direct stomach tube or nasopharyngeal tube), or through other means to accomplish delivery to the small intestine. The invention further relates to diagnostic and pharmaceutical packs and kits comprising one or more containers filled with one or more of the ingredients of 25 the aforementioned compositions of the invention.

Compounds of the invention which are Ferroportin1 proteins, Ferroportin1 fusion proteins, Ferroportin1 mimetics, *ferroportin1* gene-specific antisense poly- or oligonucleotides, inhibitors or enhancers of a Ferroportin1 may be employed alone or in

conjunction with other compounds, such as therapeutic compounds. The pharmaceutical compositions may be administered in any effective, convenient manner, including administration by topical, oral, anal, vaginal, intravenous, intraperitoneal, intramuscular, subcutaneous, intranasal, transdermal or intradermal routes, among 5 others. In therapy or as a prophylactic, the active agent may be administered to an individual as an injectable composition, for example as a sterile aqueous dispersion, preferably isotonic.

Alternatively, the composition may be formulated for topical application, for example, in the form of ointments, creams, lotions, eye ointments, eye drops, ear drops, 10 mouthwash, impregnated dressings and sutures and aerosols, and may contain appropriate conventional additives, including, for example, preservatives, solvents to assist drug penetration, and emollients in ointments and creams. Such topical formulations may also contain compatible conventional carriers, for example cream or ointment bases, and ethanol or oleyl alcohol for lotions.

15 In addition, the amount of the compound will vary depending on the size, age, body weight, general health, sex, and diet of the recipient of the compound, and the time of administration, the biological half-life of the compound, and the particular characteristics and symptoms of the disorder to be treated. Adjustment and manipulation of established dose ranges are well within the ability of those of skill in 20 the art.

A further aspect of the invention is a method to identify a polymorphism, or the presence of an alternative or variant allele of a gene in the genome of an organism (of interest here, genes encoding Ferroportin1 proteins). As used herein, polymorphism refers to the occurrence of two or more genetically determined alternative sequences or 25 alleles in a population. A polymorphic locus may be as small as a base pair. Polymorphic markers include restriction fragment length polymorphisms, variable number of tandem repeats (VNTR's), hypervariable regions, minisatellites, dinucleotide repeats, trinucleotide repeats, tetranucleotide repeats, simple sequence repeats, and

insertion elements such as Alu. The first identified allelic form, or the most frequently occurring form can be arbitrarily designated as the reference (usually, "wildtype") form, and other allelic forms are designated as alternative (sometimes, "mutant" or "variant"). Diploid organisms may be homozygous or heterozygous for allelic forms.

5 An "allele" or "allelic sequence" is an alternative form of a gene which may result from at least one mutation in the nucleotide sequence. Alleles may result in altered mRNAs or polypeptides whose structure or function may or may not be altered. Any given gene may have none, one, or many allelic forms (polymorphism). Common mutational changes which give rise to alleles are generally ascribed to natural deletions, 10 additions, or substitutions of nucleotides. Each of these types of changes may occur alone, or in combination with the others, one or more times in a given sequence.

Several different types of polymorphisms have been reported. A restriction fragment length polymorphism (RFLP) is a variation in DNA sequence that alters the length of a restriction fragment (Botstein *et al.*, *Am. J. Hum. Genet.* 32:314-331 (1980)). 15 The restriction fragment length polymorphism may create or delete a restriction site, thus changing the length of the restriction fragment. RFLPs have been widely used in human and animal genetic analyses (see WO 90/13668; WO 90/11369; Donis-Keller, *Cell* 51:319-337 (1987); Lander *et al.*, *Genetics* 121:85-99 (1989)). When a heritable trait can be linked to a particular RFLP, the presence of the RFLP in an individual can 20 be used to predict the likelihood that the individual will also exhibit the trait.

Other polymorphisms take the form of short tandem repeats (STRs) that include tandem di-, tri- and tetra-nucleotide repeated motifs. These tandem repeats are also referred to as variable number tandem repeat (VNTR) polymorphisms. VNTRs have been used in identity and paternity analysis (US 5,075,217; Armour *et al.*, *FEBS Lett.* 25 307:113-115 (1992); Horn *et al.*, WO 91/14003; Jeffreys, EP 370,719), and in a large number of genetic mapping studies.

Other polymorphisms take the form of single nucleotide variations between individuals of the same species. Such polymorphisms are far more frequent than

RFLPs, STRs (short tandem repeats) and VNTRs (variable number tandem repeats). Some single nucleotide polymorphisms occur in protein-coding sequences, in which case, one of the polymorphic forms may give rise to the expression of a defective or other variant protein and, potentially, a genetic disease. Other single nucleotide polymorphisms occur in noncoding regions. Some of these polymorphisms may also result in defective protein expression (e.g., as a result of defective splicing). Other single nucleotide polymorphisms have no phenotypic effects.

Many of the methods described below require amplification of DNA from target samples and purification of the amplified products. This can be accomplished by PCR, for instance. See generally, *PCR Technology, Principles and Applications for DNA Amplification* (ed. H.A. Erlich), Freeman Press, New York, NY, 1992; *PCR Protocols: A Guide to Methods and Applications* (eds. Innis, et al.), Academic Press, San Diego, CA, 1990; Mattila *et al.*, *Nucleic Acids Res.* 19:4967 (1991); Eckert *et al.*, *PCR Methods and Applications* 1:17 (1991); *PCR* (eds. McPherson *et al.*, IRS Press, Oxford); and US 4,683,202.

Other suitable amplification methods include the ligase chain reaction (LCR) (see Wu and Wallace, *Genomics* 4:560 (1989); Landegren *et al.*, *Science* 241:1077 (1988)), transcription amplification (Kwoh *et al.*, *Proc. Natl. Acad. Sci. USA* 86:1173 (1989), self-sustained sequence replication (Guatelli *et al.*, *Proc. Natl. Acad. Sci. USA* 87:1874 (1990), and nucleic acid based sequence amplification (NASBA). The latter two amplification methods involve isothermal reactions based on isothermal transcription, which produce both single stranded RNA (ssRNA) and double stranded DNA (dsDNA) as the amplification products in a ratio of about 30 or 100 to 1, respectively.

Another aspect of the invention is a method for detecting a variant allele of a human *ferroportin1* gene, comprising preparing amplified, purified *ferroportin1* DNA from a reference human and amplified, purified, *ferroportin1* DNA from a "test" human to be compared to the reference as having a variant allele, using the same or comparable

amplification procedures, and determining whether the reference DNA and test DNA differ in DNA sequence in the *ferroportin1* gene, whether in a coding or a noncoding region, wherein, if the test DNA differs in sequence from the reference DNA, the test DNA comprises a variant allele of a human *ferroportin1* gene. The following is a
5 discussion of some of the methods by which it can be determined whether the reference *ferroportin1* DNA and test *ferroportin1* DNA differ in sequence.

Direct Sequencing. The direct analysis of the sequence of variant alleles of the present invention can be accomplished using either the dideoxy chain termination method or the Maxam and Gilbert method (see Sambrook *et al.*, *Molecular Cloning: A
10 Laboratory Manual*, 2nd ed., Cold Spring Harbor Press, New York 1989; Zyskind *et al.*, *Recombinant DNA Laboratory Manual*, Acad. Press, 1988).

Denaturing Gradient Gel Electrophoresis. Amplification products generated using the polymerase chain reaction can be analyzed by the use of denaturing gradient gel electrophoresis. Different alleles can be identified based on the different sequence-
15 dependent strand dissociation properties and electrophoretic migration of DNA in solution (chapter 7 in Erlich, ed. *PCR Technology, Principles and Applications for DNA Amplification*, W.H. Freeman and Co., New York, 1992).

Single-strand Conformation Polymorphism Analysis. Alleles of target sequences can be differentiated using single-strand conformation polymorphism
20 analysis, which identifies base differences by alteration in electrophoretic migration of single stranded PCR products, as described in Orita *et al.*, *Proc. Natl. Acad. Sci. USA* 86:2766-2770 (1989). Amplified PCR products can be generated as described above, and heated or otherwise denatured, to form single-stranded amplification products.

Single-stranded nucleic acids may refold or form secondary structures which are
25 partially dependent on the base sequence. The different electrophoretic mobilities of single-stranded amplification products can be related to base-sequence differences between alleles of target sequences.

Detection of Binding by Protein That Binds to Mismatches. Amplified DNA comprising the *ferroportin1* gene or portion of the gene of interest from genomic DNA, for example, of a normal individual is prepared, using primers designed on the basis of the DNA sequences provided herein. Amplified DNA is also prepared, in a similar 5 manner, from genomic DNA of an individual to be tested for bearing a distinguishable allele. The primers used in PCR carry different labels, for example, primer 1 with biotin, and primer 2 with ³²P. Unused primers are separated from the PCR products, and the products are quantitated. The heteroduplexes are used in a mismatch detection assay using immobilized mismatch binding protein (MutS) bound to nitrocellulose. The 10 presence of biotin-labeled DNA wherein mismatched regions are bound to the nitrocellulose via MutS protein, is detected by visualizing the binding of streptavidin to biotin. See WO 95/12689. MutS protein has also been used in the detection of point mutations in a gel-mobility-shift assay (Lishanski, A. *et al.*, *Proc. Natl. Acad. Sci. USA* 91:2674-2678 (1994)).

15 Other methods, such as those described below, can be used to distinguish a *ferroportin1* allele from a reference allele, once a particular allele has been characterized as to DNA sequence.

Allele-specific probes. The design and use of allele-specific probes for analyzing polymorphisms is described by e.g., Saiki *et al.*, *Nature* 324:163-166 (1986); 20 Dattagupta, EP 235,726, Saiki, WO 89/11548. Allele-specific probes can be designed so that they hybridize to a segment of a target DNA from one individual but do not hybridize to the corresponding segment from another individual due to the presence of different polymorphic forms in the respective segments from the two individuals. Hybridization conditions should be sufficiently stringent that there is a significant 25 difference in hybridization intensity between alleles, and preferably an essentially binary response, whereby a probe hybridizes to only one of the alleles. Some probes are designed to hybridize to a segment of target DNA such that the polymorphic site aligns with a central position (e.g., in a 15-mer at the 7 position; in a 16-mer, at either the 8 or

9 position) of the probe. This design of probe achieves good discrimination in hybridization between different allelic forms.

Allele-specific probes are often used in pairs, one member of a pair showing a perfect match to a reference form of a target sequence and the other member showing a 5 perfect match to a variant form. Several pairs of probes can then be immobilized on the same support for simultaneous analysis of multiple polymorphisms within the same target sequence.

Allele-specific Primers. An allele-specific primer hybridizes to a site on target DNA overlapping a polymorphism, and only primes amplification of an allelic form to 10 which the primer exhibits perfect complementarity. See Gibbs, *Nucleic Acid Res.* 17:2427-2448 (1989). This primer is used in conjunction with a second primer which hybridizes at a distal site. Amplification proceeds from the two primers, resulting in a detectable product which indicates the particular allelic form is present. A control is usually performed with a second pair of primers, one of which shows a single base 15 mismatch at the polymorphic site and the other of which exhibits perfect complementarity to a distal site. The single-base mismatch prevents amplification and no detectable product is formed. The method works best when the mismatch is included in the 3'-most position of the oligonucleotide aligned with the polymorphism because this position is most destabilizing to elongation from the primer (see, e.g., WO 20 93/22456).

Gene Chips. Allelic variants can also be identified by hybridization to nucleic acids immobilized on solid supports (gene chips), as described, for example, in WO 95/11995 and U.S. Patent No. 5,143,854, both of which are incorporated herein by reference. WO 95/11995 describes subarrays that are optimized for detection of a 25 characterized variant allele. Such a subarray contains probes designed to be complementary to a second reference sequence, which is an allelic variant of the first reference sequence.

EXAMPLES

Methods

Zebrafish Strains and Studies

Linkage analysis was performed on haploid or diploid embryos obtained from 5 AB/DAR, AB/SJD or AB/WIK hybrids (Westerfield, M., *The Zebrafish Book*, Univ. Oregon Press, Eugene, 1993). Wright-Giemsa and *o*-dianisidine staining of embryos were performed as described (Ransom, D.G., *et al.*, *Development*, 123:311-319, 1996). *In situ* hybridization analysis was performed as described (Thompson, M.A., *et al.*, *Dev. Biol.*, 197:248-269, 1998).

10 Genetic Mapping and Genotyping and Library Screens

Linkage to centromeric markers (Knapik, E.W., *et al.*, *Nature Genet.*, 18:338-343, 1998) was performed by half-tetrad analysis (Johnson, S.L., *et al.*, *Genetics*, 139:1727-1735, 1995). For fine genetic mapping, haploids were genotyped on the proximal side of the locus with one of the following RAPD markers (Operon 15 Technologies, Alemeda, CA); 4W1600, 6Q1300, or 4AC800, and on the distal side with the markers 4K1300 or O61020. Diploid mutant embryos were genotyped on the proximal side with the microsatellite markers z8505 or z9479 and on the distal side with z8363 (Shimoda, N., *et al.*, *Genomics*, 58:219-232, 1999). Library screens were performed as described (Brownlie, A., *et al.*, *Nature Genet.*, 20:244-250, 1998).

20 *In situ* hybridization and rescue experiment embryos (see below) were genotyped using allele specific oligonucleotide (ASO) hybridization assays specific to the *weh*^{tp85c} and *weh*^{th238} *ferroportin1* alleles (Farr, C.J., *et al.*, *Proc. Natl. Acad. Sci. USA*, 85:1629-1633, 1988; Wood, W.I., *et al.*, *Proc. Natl. Acad. Sci. USA*, 82:1585-1588, 1985). The *weh*^{th238} oligonucleotides were developed to distinguish the C to A 25 mutation in the *weh*^{th238} allele from the wild-type allele. Wild-type is 5'-AAAGAAGTGCGGCCTCATC-3' (SEQ ID NO:8) and mutant *weh*^{th238} allele is 5'-AAAGAAGTGAGGCCTCATC-3' (SEQ ID NO: 9). The *weh*^{tp85c} oligonucleotides

were developed to distinguish the G to T mutation in the *weh*^{tp85c} allele from wild-type. Wild-type is 5'-GAGCAAATTGGCAGGTAAG-3' (SEQ ID NO:10) and mutant *weh*^{tp85c} allele is 5'-GAGCAAATTTGCAGGTAAG -3' (SEQ ID NO:11).

Isolation of the Mouse and Human *Ferroportin1* cDNAs

5 EST clones were identified that contained the 5' end (GenBank accession # D632209) and 3' end (GenBank accession # W23461) of human *ferroportin1* and the 3' end of mouse *ferroportin1* (GenBank accession # AA500296). The coding region of human and mouse *ferroportin1* cDNAs were cloned by RT-PCR with a forward primer made to the conserved iron response element (IRE) sequence in the 5' untranslated 10 region (5'-CAACTTCAGCTACAGTGTTAG-3' (SEQ ID NO:12)) and a reverse primer just 3' of the stop codon of each cDNA (mouse 5'-TTATACAAACAGATGTATTCGGT-3' (SEQ ID NO:13) and human 5'-AACTGTCTCAAACAAACAGATG-3' (SEQ ID NO:14)).

Embryo Injection Experiments

15 A zebrafish Ferroportin1-GFP fusion protein construct was created by PCR. The forward PCR primer contained the start codon, 5'-CCGCTCGAGAACGCACAATGGACAGCCCTG-3' (SEQ ID NO:15). The reverse primer contained the last codon, 5'-CCGCTCGAGTACAGAGTTGGAAGTGAGGG-3' (SEQ ID NO:16). The PCR 20 product was subcloned into the GFP expression vector pEGFP-N1 (Clontech, Palo Alto, CA). Embryos from a cross of two *weh*^{th238} heterozygotes were injected (Westerfield, M., *The Zebrafish Book*, Univ. Oregon Press, Eugene, 1993) with the *ferroportin1*-GFP plasmid (300 ng/ml). For the iron-dextran rescue experiment, 48 hr mutant embryos 25 from a *weh*^{tp85c} cross were injected intravenously with an iron-dextran solution (Sigma, 100 mg/ml).

Xenopus oocyte injections and ⁵⁵Fe efflux experiments

cRNA for injection was prepared using the mMessage Machine kit (Ambion, Austin, TX), using a construct containing either the rat *DMT1* cDNA in pSPORT1 (gift from Hiromi Gunshin (Gunshin, H., *et al.*, *Nature*, 388:482-488, 1997)) or the zebrafish *ferroportin1* cDNA in the vector pXT7 (kindly provided by Sergei Sokol).

5 Defollicularized oocytes were incubated in ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM Hepes, and 2.5 mM sodium pyruvate, pH 7.4) and injected with either 20 ng of *DMT1* cRNA alone or 20 ng each of both the *DMT1* and *ferroportin1* cRNAs. ⁵⁵Fe uptake and efflux were performed 48 hrs after injection. ⁵⁵Fe uptake was performed in 500 µl of a solution containing 60 µM ⁵⁵FeCl₂, 100 mM NaCl, 10 mM Hepes and 1 mM ascorbic acid at pH 5.5 for 30 minutes. ⁵⁵Fe uptake was stopped by incubation of the oocytes in 1 mM cold FeCl₂, 100 mM NaCl, 10 mM Hepes, and 1 mM ascorbic acid at pH 6.0 for 30 minutes. Individual oocytes were placed in 500 µl of either efflux buffer (100 mM NaCl, 10 mM Hepes at pH 7.4) alone or efflux buffer containing a final concentration of 20 mg/ml apo-transferrin for 60

10 minutes. After incubation, the ⁵⁵Fe levels of both the efflux solution and the individual oocytes lysed in 10% SDS were measured by scintillation counting.

15

Mouse *in situ* hybridization and Northern Blot Analysis

In *situ* hybridization of murine embryos was performed as described (Palis, J., *et al.*, *Mol. Reprod. Dev.*, 42:19-27, 1995). An adult multi-tissue Human Northern blot (Clontech, Palo Alto CA) containing 2 µg of poly A⁺ mRNA per lane was probed with a human *ferroportin1* EST (GenBank accession # W05488). The mouse intestinal Northern blot containing 20 µg of total RNA per lane was probed with a mouse *ferroportin1* EST (GenBank accession # AA500296).

25 Antibody Generation and Immunohistochemistry

A rabbit polyclonal antibody was generated to a peptide consisting of the C-terminal 19 amino acids of the human Ferroportin1 protein (Genemed Synthesis, San

Francisco CA). Antiserum was affinity-purified against the peptide. Formalin or Bouin's fixed paraffin-embedded specimens were deparaffinized and heat treated for 30 min in 1.0 mM EDTA, pH 8.0, in a Black and Decker steamer (Model HS80). Endogenous peroxidase activity was quenched in methanol and 3% hydrogen peroxide (5:1, vol/vol).

- 5 The slides were then incubated in 3% normal swine serum in 0.05 M Tris, pH 7.6, followed by rabbit anti-Ferroportin1 antibody (7 µg/ml), and sequentially in HRP-conjugated goat anti-rabbit immunoglobulins (1:40 dilution, Dako), HRP-conjugated rabbit anti-goat immunoglobulins (1:40 dilution, Dako), followed by HRP-conjugated swine anti-rabbit immunoglobulins (1:52 dilution, Dako), each
- 10 prepared in 0.10 M Tris, pH 7.6, containing (4%) human AB serum. Antibody localization was determined using DAB in 0.5 M Tris (pH 7.6) containing (0.035%) hydrogen peroxide. Slides were counterstained with methyl green. Control samples were incubated with normal rabbit serum or purified rabbit immunoglobulins at a protein concentration equal to the antibody preparation. In addition, preincubation of the
- 15 antibody with specific peptide at a 550-fold molar excess neutralized the reactivity.

Red Cell Iron Measurement

Iron levels in red blood cells were measured by Atomic Absorption Spectrometer Model 3030 equipped with Zeeman Graphite Furnace and Autosampler, (Perkin Elmer Corp.). A cell sample (at least 30,000 cells in 20 µl PBS) or blank (PBS) was diluted with 40 µl of 480 mg/dl of magnesium nitrate (matrix modifier); 20 µl of the mixture was injected into the instrument and analyzed in duplicate. The instrument was calibrated with iron standards of 10, 25, 100 and 250 ng/ml prepared from Atomic Spectroscopy Standard (Perkin Elmer Corp.).

Structure Prediction

- 25 Hydropathy plots (Kyte-Doolittle) were obtained using the Genetics Computer Group (GCG) programs PEPTIDESTRUCTURE and PEPPLOT with an hydropathy

window of 14. Transmembrane amino acid segments were identified and their topography predicted using the programs PHDhtm (www.embl-heidelberg.de/predictprotein/predictprotein.html), HMMTOP (www.enzim.hu/hmmtop/), TMHMM (www.cbs.dtu.dk/services/TMHMM-1.0/),
5 TMpred (www.ch.embnet.org/software/TMPRED_form.html), TopPred2 (www.biokemi.su.se/~server/toppred2/), and SOSUI (www.tuat.ac.jp/~mitaku/). Most analyses predict 10 TM segments. Some predict that the first and last TM segments are split, and that Ferroportin contains 12 segments. Others predict that TM segments 4 and 5 are not split and only 9 segments are present.

10 Example 1: Observations on *weissherbst* Zebrafish Mutants

Two independent autosomal recessive mutations of the zebrafish hypochromic blood mutant *weissherbst* (*weh*), *weh*^{th238} and *weh*^{tp85c}, were isolated as part of a large-scale screen for ethyl nitroso urea (ENU) induced mutations that disrupt embryonic development in zebrafish (Haffter, P., *et al.*, *Danio rerio. Development*,
15 123:1-36, 1996). These *weh* mutants were identified in a morphologic screen for defects in circulating erythroid cells (Ransom, D.G., *et al.*, *Development*, 123:311-319, 1996). While the number of circulating erythroid cells of both mutant alleles are normal at 33 and 48 hours post fertilization (hpf), the *weh* mutant cells are hypochromic (lacking red color). Mutant embryos show little, if any, hemoglobin compared to
20 wild-types at 33 hpf and 48 hpf (by *o*-dianisidine staining), but some hemoglobin is detectable at 72 hpf. The *weh*^{th238} allele has less *o*-dianisidine staining compared to the *weh*^{tp85c} allele at 72 hpf, suggesting that it is a more severely defective allele. In addition to the hypochromia, a progressive decrease in red cell number occurs after 48 hpf. By 96 hpf, the number of circulating erythroid cells in mutants decreases to approximately
25 20% of the number in wild-type. The *weh* mutant cells at days 2 (56 hpf) and 3 (80 hpf) have a large nucleus and basophilic cytoplasm characteristic of more immature erythroid cells, and on day 5 (125 hpf) the remaining mutant cells are misshapen. Further studies

of erythroid differentiation reveal that embryonic globin mRNA levels are abnormally maintained during maturation. Although *weh* mutants have no gross organ defects in addition to their anemia, all mutant embryos die between day 7 and day 14 of development.

Given the possibility that hypochromia could result from iron deficiency, we measured iron levels in *weh* erythroid cells. The level of iron measured in 10^4 wild-type cells ranged from 0.093 ng to 0.208 ng (n=3), whereas the levels of iron in the same number of *weh* mutant cells ranged from 0.014 ng to 0.033 ng (n=3). The 4-9 fold decrease in erythrocyte iron levels could be due to low levels of iron in circulation. As confirmation of this hypothesis, iron-dextran injected intravenously into *weh*^{tp85c} mutant embryos was shown to completely rescue hemoglobin production as seen by *o*-diansidine staining of hemoglobin. This rescue demonstrated that *weh* mutant erythroid cells are fully capable of hemoglobinization, and that the hypochromia is caused by inadequate circulatory iron levels.

15 Example 2: Isolation of *weh* Mutant Gene

To gain further insight into this phenotype, we isolated the *weh* mutant gene by positional cloning methods. Study of the segregation of centromeric microsatellite markers in half-tetrad gynogenetic diploid embryos localized *weh* to linkage group 9. Genetic mapping placed the *weh* locus in an approximately 6 cM interval between the random amplified polymorphic DNA (RAPD) markers 4K1300 and 6Q1300 (Fig. 1). We isolated more closely linked markers using amplified fragment length polymorphism (AFLP) analysis (Ransom, D.G., *et al.*, pp. 195-210 in *The Zebrafish: Genetics and Genomics*, eds. Detrich, H.W.L., Westerfield, M. & Zon, L.I., Academic, San Diego, 1999; Vos. P., *et al.*, *Nucleic Acids Res.*, 23:4407-4414, 1995), scanning approximately 10,000 polymorphic loci. Single strand conformational polymorphism (SSCP) analysis showed that the AFLP marker I36 was 0.13 cM proximal to the *weh* locus (Fig. 1).

A chromosomal walk towards the gene was initiated from the I36 marker (Fig. 1), resulting in the identification of a critical interval that contained the *weh* gene (Fig. 1). In an attempt to identify potential candidates for the *weh* gene, we used a hybridization strategy to screen cDNA libraries for genes located on the PAC clones identified in the region of the *weh* locus. We hybridized a radiolabelled insert of PAC clone 170G3 to zebrafish gridded cDNA libraries. Screening of 100,000 gridded cDNA clones identified 5 clones of a novel cDNA, designated *WC1* for *weh* cDNA #1. Analysis of *WC1* mRNA expression in embryos and sequencing *WC1* from *weh*^{th238} mutants suggested that *WC1* was not a candidate for *weh*. Two genes, *STAT1* and *glutaminase*, were isolated by hybridization of the zebrafish PAC clone 87I4 (Fig. 1) to gridded cDNA libraries. The human orthologs of *WC1*, *STAT1* and *glutaminase* are localized to human chromosome 2 in a 2.4 cM interval, demonstrating conserved chromosomal synteny among vertebrates (Postlethwait, J.H., *et al.*, *Nature Genet.*, 18:345-349, 1998). Homology searches identified a pufferfish (*Fugu rubripes*) cosmid clone (121D21) that contained both *WC1* and *STAT1*. This cosmid also contained Fugu homologs of other genes located on human chromosome 2. Using primers designed to the pufferfish sequence of one of these genes (121D21aB3), a 200 bp fragment of the zebrafish ortholog was amplified from PAC 211013. A full length cDNA (3.7 kb) of this gene, hereafter referred to as *ferroportin1*, was isolated from a zebrafish kidney cDNA library.

This gene, *ferroportin1*, has a predicted open reading frame of 562 amino acids (Fig. 2). Sequence analysis of the *weh*^{th238} allele identified a C to A nucleotide transversion that causes premature termination of translation at codon 361 (Fig. 2). Similar analysis of the *weh*^{tp85c} allele identified a single amino acid change, Leu168Phe, resulting from a G to T nucleotide difference (Fig. 2). The finding of a premature stop mutation in *weh*^{th238} strongly suggests that the *weh* mutant phenotype is caused by a defect in *ferroportin1*. Mouse and human *ferroportin1* cDNA clones were obtained by RT-PCR of RNA isolated from liver and placenta, respectively. A conserved sequence,

predicted to form a hairpin loop structure typical of iron response elements (IREs) (Eisenstein, R.S., *et al.*, *J. Nutr.*, 128:2295-2298, 1998), was identified in the 5' untranslated region (UTR) of the cDNAs from all three species. Based on protein structure prediction analysis, Ferroportin1 contains at least 10 transmembrane segments

5 (Fig. 2).

Example 3: Expression of *ferroportin1*

In situ hybridization analysis of zebrafish embryos shows that *ferroportin1* mRNA is not expressed in erythroid cells. *Ferroportin1* mRNA is detected at 18 hpf through 48 hpf in the yolk syncytial layer (YSL). The YSL is the peripheral layer of the yolk cell that lies just below the membrane (Kimmel, C.B., *et al.*, *Dev. Dyn.*, 203:253-310, 1995). This layer surrounds the entire yolk of the embryo and consists of yolk-free cytoplasm and nuclei. Yolk has been shown to contain nutrients needed during development (Richards, M.P., *Poult. Sci.*, 76:152-164, 1997), including iron (Richards, M.P., *Poult. Sci.*, 76:152-164, 1997; Dumont, J.N., *J. Exp. Zool.*, 204:193-217, 1978; Craik, J.C., *Comp. Biochem. Physiol. A.*, 83:515-517, 1986). Embryos express *ferroportin1* in the region of the YSL at 18 hpf that lies just below the developing hematopoietic cells in the intermediate cell mass (Al-Adhami, M.A., *et al.*, *Develop. Growth Differ.*, 19:171-179, 1977). At 48 hpf, *ferroportin1* is expressed in the brain and in a localized region of the YSL. Note that expression is in the same region of the YSL over which the blood flows (Reib, J.P., *Annales D'Embryologie et de Morphogenese*, 6:43-54, 1973). At both time-points, *ferroportin1* is expressed in the region of the YSL adjacent to the blood, but not by the entire YSL. This pattern of expression suggests that *ferroportin1* expression and function in the YSL is linked to red blood cell development. Considering the iron-dextran rescue of hemoglobin production in *weh* mutants, the YSL expression of *ferroportin1* suggested that the gene might function in the transport of iron from the yolk to the embryonic circulation.

To provide evidence that defects in the *ferroportin1* gene cause the *weh* mutant phenotype, we injected a plasmid that expresses Ferroportin1 fused to green fluorescent protein (GFP) into the yolk cell between the 256 and 1000 cell stages (Kimmel, C.B., *et al.*, *Dev. Dyn.*, 203:253-310, 1995). At 48 hours of development, 33% of injected 5 embryos expressed GFP strictly in the YSL. At 80 hpf, the phenotype of mutant embryos expressing GFP was compared to the uninjected mutants. The Ferroportin1-GFP-expressing mutant embryos (n=9) had considerably more hemoglobin expression than uninjected mutants as observed by *o*-dianisidine staining. This partial rescue of the hypochromia provides further evidence that *ferroportin1* is the *weh* gene, 10 and demonstrates that Ferroportin1 acts in the YSL. The rescue of the *weh* mutant phenotype by intravenous iron-dextran injection and by Ferroportin1 expressed in the YSL indicates that Ferroportin1 functions to deliver yolk iron into the embryonic circulation.

The function of Ferroportin1 was tested using a *Xenopus* oocyte expression 15 system. Since the proposed function of Ferroportin1 is to export iron, it was necessary to first load the oocytes with ^{55}Fe . Radioactive iron loading was accomplished through the expression of the iron transporter DMT1 in oocytes and then loading of ^{55}Fe at pH 5.5. ^{55}Fe loaded oocytes that expressed either DMT1 alone, or DMT1 and Ferroportin1, were tested for iron export activity either in the presence or absence of apo-transferrin, 20 an iron chelator. To normalize for the iron content in each individual oocyte, the ratio of efflux to uptake was calculated. Our results showed that, in the presence of apo-transferrin, the efflux to uptake ratio in oocytes expressing Ferroportin1 was five fold greater than control oocytes not expressing Ferroportin1 (Fig. 3, $P << 0.001$).

Example 4: Expression of *ferroportin1* in Embryonic and Adult Tissues of Mammals

25 To evaluate a potential role for *ferroportin1* in iron transport in mammals, we examined tissue expression. Northern blot analysis showed highest levels of expression in human placenta, liver, spleen, and kidney. In mice, *ferroportin1* mRNA is expressed

specifically in the duodenum but not in the jejunum or ileum. Additionally, *ferroportin1* is expressed in the large intestine. Most intestinal iron absorption occurs in the proximal duodenum, placing *ferroportin1* in a physiologically appropriate location to play a role in intestinal iron absorption. mRNA *in situ* hybridization analysis was

5 performed on sections of mouse embryos. The primitive erythroblasts derived from the blood islands do not express *ferroportin1*, whereas the trophoblast cells of the inner placenta express high levels of *ferroportin1* RNA. *Ferroportin1* transcripts were found in the inner placenta (labyrinth zone) and the trophoblast giant cells at the border between the outer placenta (spongiotrophoblast), but not in the maternal deciduum.

10 *Ferroportin1* expression is also present in the visceral endoderm of the yolk sac surrounding the embryo proper. Within the embryo proper, *ferroportin1* transcripts were detected in several tissues, including the vascular plexus surrounding the central nervous systems, but particularly the gut and liver. The expression of *ferroportin1* in placenta, duodenum, and liver, all prominent sites of iron transport, is consistent with

15 the proposed role of the gene in iron transport.

In order to characterize the expression of Ferroportin1 protein, we generated a specific rabbit polyclonal antibody against a *Ferroportin1* peptide. In the human placenta, Ferroportin1 protein was primarily expressed in a basal location within the syncytiotrophoblasts. The basal surface of the syncytiotrophoblast interfaces with the

20 fetal circulation, whereas the apical surface contacts the maternal circulation. The mammalian placenta and the zebrafish YSL provide a homologous function, serving as the site of iron transfer between mother and embryo. Taken together with the functional data in zebrafish and *Xenopus*, Ferroportin1 is likely to export iron from the syncytiotrophoblast into the embryonic circulation.

25 A similar analysis of mouse duodenum showed Ferroportin1 staining in enterocytes in the villus. The intensity of staining was stronger at the tip of the villus compared to the crypt. Staining was particularly strong at the basolateral surface of the enterocyte. The duodenal enterocytes of the small intestine are polarized epithelial cells

that transport iron into the intestinal capillaries through the basolateral membrane. The mechanism of intestinal basolateral iron transport has not been established. Sex-linked anemia (*sla*) mice have a defect in basolateral iron transport in the duodenum, based on ferrokinetic studies and the presence of abnormal iron deposits in duodenal enterocytes

5 (Bannerman, R.M., *Fed. Proc.*, 35:2281-2285, 1976). Analogous to *weh* mutants, the *sla* mouse has a defect in transport of maternal iron to the embryonic circulation (Kingston, P.J., *et al.*, *Br. J. Haematol.*, 40:265-276, 1978). The *sla* phenotype is due to a mutation in the membrane-bound multi-copper ferroxidase gene, *hephaestin* (Vulpe, C.D., *et al.*, *Nature Genet.*, 21:195-199, 1999). In *Saccharomyces cerevisiae*, the

10 hephaestin-like ferroxidase *FET3* is required for high affinity iron uptake by the iron transporter *FTR1* (Askwith, C., *et al.*, *Cell*, 76:403-410, 1994; Stearman, R., *et al.*, *Science*, 271:1552-1557, 1996). Expression of Ferroportin1 at the basolateral surface of duodenal enterocytes in mouse and the multiple-transmembrane structure of the protein make it an excellent candidate to function as a basolateral iron transporter.

15 Additional data from the *weh* mutant suggests that Ferroportin1 functions in the intestine of the adult zebrafish. Both *in situ* hybridization studies and immunohistochemistry showed expression of zebrafish *ferroportin1* in the intestine. In addition, iron-dextran rescued mutant embryos live past the normal time of lethality (day 7-14). We have successfully raised these rescued embryos to adulthood. These

20 fish are smaller than their wildtype siblings and have a profound hypochromic anemia. Since these fish eat a normal diet replete in iron, but nonetheless are severely anemic, these data suggest that the gene is required for intestinal iron absorption in addition to yolk sac transport. Based on the basolateral expression pattern of Ferroportin1 in mammalian enterocytes and the implication that *ferroportin1* is required for intestinal

25 iron transport in zebrafish, it is likely that the protein is involved in iron export from enterocytes in mammals. Further experiments are required to determine whether Ferroportin1 cooperates with hephaestin in these cells.

Other tissues may utilize *ferroportin1* as an iron exporter. Very high levels of expression are evident in Kupffer cells of human liver, the resident macrophages of the liver, and macrophages located within the splenic red pulp. Hepatocytes were also positive by immunohistochemical staining. Ferroportin1 could play a role in iron export from macrophages, a critical function in recycling of iron from senescent erythrocytes.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

FERROPORTIN1 NUCLEIC ACIDS AND PROTEINS

ABSTRACT OF THE DISCLOSURE

Positional cloning has been carried out to identify the gene responsible for the hypochromic anemia of the zebrafish mutant *weissherbst*. The gene, *ferroportin1*, 5 encodes a novel multiple-transmembrane domain protein, expressed in the yolk sac. Zebrafish *ferroportin1* is required for the transport of iron from maternally-derived yolk stores to the circulation, and functions as an iron exporter when expressed in *Xenopus* oocytes. Human and mouse homologs of the *ferroportin1* gene have been identified. The invention includes isolated polynucleotides, vectors and host cells comprising 10 nucleotide sequences encoding Ferroportin1 proteins and variants thereof, including those having iron transport function. The invention also includes polypeptides encoded by *ferroportin1* genes and variants of such polypeptides, and fusion polypeptides comprising a Ferroportin1 or a portion thereof. Methods to produce a Ferroportin1, methods to produce antibodies to a Ferroportin1 and methods to identify agents binding 15 to a Ferroportin1, which can be inhibitors or enhancers of Ferroportin1 iron transport activity, are also described. Inhibitors of Ferroportin1 activity can be used in a therapy for hemochromatosis.

CLAIMS

What is claimed is:

1. An isolated nucleic acid comprising the nucleotide sequence SEQ ID NO:1.
- 5 2. An isolated nucleic acid having at least 80% nucleotide sequence similarity to a nucleic acid encoding a polypeptide comprising the amino acid sequence SEQ ID NO:2.
- 10 3. An isolated nucleic acid comprising a nucleotide sequence which consists of the coding region of zebrafish *ferroportin1*.
- 15 4. An isolated nucleic acid comprising a nucleotide sequence which encodes a contiguous portion of at least about 15 amino acids of SEQ ID NO:2.
5. An isolated nucleic acid comprising a nucleotide sequence which encodes a naturally occurring allelic variant of a polypeptide consisting of the amino acid sequence of a zebrafish Ferroportin1 protein, wherein said nucleic acid molecule hybridizes under high stringency conditions to the complement of the sequence SEQ ID NO:1.
- 20 6. An isolated nucleic acid molecule which encodes a polypeptide having an iron transport function, wherein said molecule hybridizes under high stringency conditions to SEQ ID NO:1 or its complement.
7. An isolated nucleic acid which hybridizes under high stringency conditions to nucleic acid consisting of nucleotides 238 through 1926 within SEQ ID NO:1.

8. An isolated nucleic acid molecule encoding a fusion polypeptide, said nucleic acid molecule comprising a nucleotide sequence encoding all or a portion of an amino acid sequence SEQ ID NO:2, and further comprising a nucleotide sequence encoding a heterologous portion of said fusion polypeptide.

5 9. An isolated nucleic acid comprising a nucleotide sequence which encodes a protein comprising the amino acid sequence SEQ ID NO:2, or SEQ ID NO:2 with conservative amino acid substitutions.

10. A nucleic acid vector comprising nucleic acid having at least 80% nucleotide sequence similarity to a nucleic acid encoding a polypeptide comprising the amino acid sequence SEQ ID NO:2, operably linked to an expression control sequence.

15 11. A nucleic acid vector comprising nucleic acid encoding a fusion polypeptide, said nucleic acid molecule comprising a nucleotide sequence encoding all or a portion of an amino acid sequence SEQ ID NO:2, and further comprising a nucleotide sequence encoding a heterologous portion of said fusion polypeptide.

12. A nucleic acid vector comprising nucleic acid comprising a nucleotide sequence which encodes a protein comprising the amino acid sequence SEQ ID NO:2, or SEQ ID NO:2 with conservative amino acid substitutions.

20 13. A nucleic acid expression vector comprising a coding sequence encoding zebrafish Ferroportin1.

14. A nucleic acid vector comprising a nucleic acid with at least 80% nucleotide sequence similarity to the coding region of SEQ ID NO:1.

15. A nucleic acid vector comprising a nucleic acid encoding a fusion polypeptide, said nucleic acid comprising a nucleotide sequence which encodes a contiguous portion of at least 15 amino acids of SEQ ID NO:2.

16. A cultured cell comprising the vector of Claim 10.

5 17. A cultured cell comprising the vector of Claim 11.

18. A cultured cell comprising nucleic acid having at least 80% nucleotide sequence similarity to a nucleic acid encoding a polypeptide comprising the amino acid sequence SEQ ID NO:2.

10 19. A cultured cell comprising nucleic acid comprising a nucleotide sequence which encodes a contiguous portion of at least about 15 amino acids of SEQ ID NO:2.

20. A cultured cell comprising nucleic acid comprising a nucleotide sequence which encodes a polypeptide having an iron transport function, wherein said nucleic acid hybridizes under high stringency conditions to SEQ ID NO:1 or its complement.

15 21. A method for producing a polypeptide, said method comprising culturing the cell of Claim 18 under conditions in which the cell produces the polypeptide.

22. A method for producing a polypeptide, said method comprising culturing the cell of Claim 20 under conditions in which the cell produces the polypeptide.

23. A method for producing a polypeptide, said method comprising culturing the cell of Claim 20 under conditions in which the cell produces the polypeptide, and isolating the polypeptide from the cell.

5 24. An isolated nucleic acid comprising the nucleotide sequence SEQ ID NO:3.

25. An isolated nucleic acid having at least 80% nucleotide sequence identity to a nucleic acid encoding a polypeptide comprising the amino acid sequence SEQ ID NO:3.

10 26. An isolated nucleic acid comprising a nucleotide sequence which consists of the coding region of mouse Ferroportin1.

27. An isolated nucleic acid comprising a nucleotide sequence which encodes a contiguous portion of at least 175 amino acids of SEQ ID NO:4.

15 28. An isolated nucleic acid consisting of a sequence of at least 510 contiguous nucleotides complementary to a region between nucleotides 298 and 2010 of SEQ ID NO:3.

29. An isolated nucleic acid comprising a nucleotide sequence which encodes a naturally occurring allelic variant of a polypeptide consisting of the amino acid sequence of a Ferroportin1 protein, wherein said nucleic acid molecule hybridizes under high stringency conditions to the complement of the sequence SEQ ID NO:3.

30. An isolated nucleic acid which encodes a polypeptide having an iron transport function, wherein said molecule hybridizes under high stringency conditions to SEQ ID NO:3 or its complement.

31. An isolated nucleic acid encoding a fusion polypeptide, said nucleic acid molecule comprising a nucleotide sequence encoding all or a portion of an amino acid sequence SEQ ID NO:4, and further comprising a nucleotide sequence encoding a heterologous portion of said fusion polypeptide.

5 32. An isolated nucleic acid comprising a nucleotide sequence which encodes a protein comprising the amino acid sequence SEQ ID NO:4, or SEQ ID NO:4 with conservative amino acid substitutions.

10 33. A nucleic acid vector comprising nucleic acid having at least 80% nucleotide sequence identity to a nucleic acid encoding a polypeptide comprising the amino acid sequence SEQ ID NO:4.

15 34. A nucleic acid vector comprising nucleic acid encoding a fusion polypeptide, said nucleic acid molecule comprising a nucleotide sequence encoding all or a portion of an amino acid sequence SEQ ID NO:4, and further comprising a nucleotide sequence encoding a heterologous portion of said fusion polypeptide.

20 35. A nucleic acid vector comprising nucleic acid comprising a nucleotide sequence which encodes a protein comprising the amino acid sequence SEQ ID NO:4, or SEQ ID NO:4 with conservative amino acid substitutions.

36. A nucleic acid expression vector comprising a coding sequence encoding mouse Ferroportin1.

37. A nucleic acid vector comprising a nucleic acid encoding a fusion polypeptide, said nucleic acid comprising a nucleotide sequence which encodes a contiguous portion of at least 15 amino acids of SEQ ID NO:4.

38. A cultured cell comprising the vector of Claim 33.

5 39. A cultured cell comprising the vector of Claim 34.

40. A cultured cell comprising nucleic acid having at least 80% nucleotide sequence similarity to a nucleic acid encoding a polypeptide comprising the amino acid sequence SEQ ID NO:4.

10 41. A cultured cell comprising nucleic acid comprising a nucleotide sequence which encodes a contiguous portion of at least about 15 amino acids of SEQ ID NO:4.

42. A cultured cell comprising nucleic acid comprising a nucleotide sequence which encodes a polypeptide having an iron transport function, wherein said nucleic acid hybridizes under high stringency conditions to SEQ ID NO:3 or its complement.

15 43. A method for producing a polypeptide, said method comprising culturing the cell of Claim 40 under conditions in which the cell produces the polypeptide.

44. A method for producing a polypeptide, said method comprising culturing the cell of Claim 42 under conditions in which the cell produces the polypeptide.

45. A method for producing a polypeptide, said method comprising culturing the cell of Claim 42 under conditions in which the cell produces the polypeptide, and isolating the polypeptide from the cell.

46. An isolated nucleic acid comprising the nucleotide sequence SEQ ID NO:5.

5 47. An isolated nucleic acid having at least 80% nucleotide sequence identity to a nucleic acid encoding a polypeptide comprising the amino acid sequence SEQ ID NO:5.

48. An isolated nucleic acid comprising a nucleotide sequence which consists of the coding region of human Ferroportin1.

10 49. An isolated nucleic acid comprising a nucleotide sequence which encodes a contiguous portion of at least 190 amino acids of SEQ ID NO:6.

50. An isolated nucleic acid consisting of a sequence of at least 575 contiguous nucleotides complementary to a region between nucleotides 305 and 2020 of SEQ ID NO:5.

15 51. An isolated nucleic acid comprising a nucleotide sequence which encodes a naturally occurring allelic variant of a polypeptide consisting of the amino acid sequence of a Ferroportin1 protein, wherein said nucleic acid molecule hybridizes under high stringency conditions to the complement of the sequence SEQ ID NO:5.

52. An isolated nucleic acid which encodes a polypeptide having an iron transport function and is at least 457 amino acid residues long, wherein said molecule hybridizes under high stringency conditions to SEQ ID NO:5 or its complement.

5 53. An isolated nucleic acid which hybridizes under high stringency conditions to nucleic acid consisting of nucleotides 305 through 2020 within SEQ ID NO:5.

54. An isolated nucleic acid comprising a nucleotide sequence encoding a polypeptide, wherein said nucleotide sequence shares at least 80% sequence identity with the nucleotide sequence SEQ ID NO:5.

10 55. An isolated nucleic acid encoding a fusion polypeptide, said nucleic acid molecule comprising a nucleotide sequence encoding all or a portion of an amino acid sequence SEQ ID NO:6, and further comprising a nucleotide sequence encoding a heterologous portion of said fusion polypeptide.

15 56. An isolated nucleic acid comprising a nucleotide sequence which encodes a protein comprising the amino acid sequence SEQ ID NO:6, or SEQ ID NO:6 with conservative amino acid substitutions.

57. A nucleic acid vector comprising nucleic acid having at least 80% nucleotide sequence identity to a nucleic acid encoding a polypeptide comprising the amino acid sequence SEQ ID NO:6.

20 58. A nucleic acid vector comprising nucleic acid encoding a fusion polypeptide, said nucleic acid molecule comprising a nucleotide sequence encoding all or a portion of an amino acid sequence SEQ ID NO:6, and further comprising a nucleotide sequence encoding a heterologous portion of said fusion polypeptide.

59. A nucleic acid vector comprising nucleic acid comprising a nucleotide sequence which encodes a protein comprising the amino acid sequence SEQ ID NO:6, or SEQ ID NO:6 with conservative amino acid substitutions.

60. A nucleic acid vector comprising nucleic acid having at least 80% nucleotide sequence identity to a nucleic acid encoding a polypeptide comprising the amino acid sequence SEQ ID NO:5.

5

61. A nucleic acid expression vector comprising a coding sequence encoding human Ferroportin1.

62. A nucleic acid vector comprising a nucleic acid with at least 80% nucleotide sequence identity to the coding region of SEQ ID NO:5.

10

63. A nucleic acid vector comprising a nucleic acid encoding a fusion polypeptide, said nucleic acid comprising a nucleotide sequence which encodes a contiguous portion of at least 15 amino acids of SEQ ID NO:6.

64. A nucleic acid vector comprising a nucleic acid encoding a fusion polypeptide, said nucleic acid comprising a nucleotide sequence encoding all or a portion of an amino acid sequence SEQ ID NO:6, and further comprising a nucleotide sequence encoding a heterologous portion of said fusion polypeptide.

15

65. A cultured cell comprising the vector of Claim 59.

66. A cultured cell comprising the nucleic acid vector of Claim 63.

67. A cultured cell comprising nucleic acid having at least 80% nucleotide sequence similarity to a nucleic acid encoding a polypeptide comprising the amino acid sequence SEQ ID NO:2.

5 68. A cultured cell comprising nucleic acid comprising a nucleotide sequence which encodes a contiguous portion of at least about 15 amino acids of SEQ ID NO:2.

69. A cultured cell comprising nucleic acid comprising a nucleotide sequence which encodes a polypeptide having an iron transport function, wherein said nucleic acid hybridizes under high stringency conditions to SEQ ID NO:1 or its complement.

10 70. A method for producing a polypeptide, said method comprising culturing the cell of Claim 67 under conditions in which the cell produces the polypeptide.

15 71. A method for producing a polypeptide, said method comprising culturing the cell of Claim 67 under conditions in which the cell produces the polypeptide, and isolating the polypeptide from the cell or the culture medium.

72. An isolated nucleic acid comprising the nucleotide sequence SEQ ID NO:7.

73. An isolated immunogenic polypeptide, the amino acid sequence of which comprises at least 19 consecutive amino acid residues of SEQ ID NO:2.

20 74. An isolated polypeptide comprising at least 10 amino acid residues of a cytoplasmic or extracellular domain of zebrafish Ferroportin1.

75. An isolated polypeptide comprising the amino acid sequence SEQ ID NO:2, or SEQ ID NO:2 with at least one conservative amino acid substitution.

76. An isolated polypeptide, the amino acid sequence of which is at least 80% identical to SEQ ID NO:2.

5 77. An isolated polypeptide, the amino acid sequence of which is at least 80% similar to SEQ ID NO:2.

78. An isolated polypeptide the amino acid sequence of which consists of SEQ ID NO:2.

79. An isolated polypeptide produced by the method of Claim 21.

10 80. An isolated polypeptide encoded by a nucleic acid molecule comprising a nucleotide sequence which encodes a naturally occurring allelic variant of a polypeptide, the polypeptide consisting of the amino acid sequence of zebrafish Ferroportin1, wherein said nucleic acid molecule hybridizes to a complement of a nucleic acid molecule consisting of SEQ ID NO:1 under high stringency conditions.

15

81. A fusion protein comprising a polypeptide selected from the group consisting of:

20 a) a naturally occurring allelic variant of a polypeptide, the polypeptide consisting of the amino acid sequence of zebrafish Ferroportin1 in SEQ ID NO:2;

b) a polypeptide consisting of an amino acid sequence which is at least 95% identical to the amino acid sequence of SEQ ID NO:2;

- c) a polypeptide consisting of amino acid sequence SEQ ID NO:2; and
- d) a polypeptide comprising a contiguous portion of at least about 15 amino acid residues of any of the foregoing.

82. The fusion protein of Claim 81 wherein the fusion protein transports iron across
5 a cell membrane or an artificial cell membrane system.

83. An isolated immunogenic polypeptide, the amino acid sequence of which comprises at least 19 consecutive amino acid residues of SEQ ID NO:4.

84. An isolated polypeptide comprising at least 10 amino acid residues of a cytoplasmic or extracellular domain of mouse Ferroportin1.

10 85. An isolated polypeptide comprising the amino acid sequence SEQ ID NO:4, or SEQ ID NO:4 with at least one conservative amino acid substitution.

86. An isolated polypeptide, the amino acid sequence of which comprises a sequence at least 80% identical to SEQ ID NO:4.

15 87. An isolated polypeptide, the amino acid sequence of which comprises a sequence at least 80% similar to SEQ ID NO:4.

88. An isolated polypeptide the amino acid sequence of which consists of SEQ ID NO:4.

89. An isolated polypeptide produced by the method of Claim 43.

90. An isolated polypeptide encoded by a nucleic acid molecule comprising a nucleotide sequence which encodes a naturally occurring allelic variant of a polypeptide, the polypeptide consisting of the amino acid sequence of mouse Ferroportin1, wherein said nucleic acid molecule hybridizes to the complement of a nucleic acid molecule consisting of SEQ ID NO:4 under high stringency conditions.

5

91. A fusion protein comprising a polypeptide selected from the group consisting of:

10

- a) a naturally occurring allelic variant of a polypeptide, the polypeptide consisting of the amino acid sequence of mouse Ferroportin1 in SEQ ID NO:4;
- b) a polypeptide consisting of an amino acid sequence which is at least 95% identical to the amino acid sequence of SEQ ID NO:4;
- c) a polypeptide consisting of amino acid sequence SEQ ID NO:4; and

15

- d) a polypeptide comprising a contiguous portion of at least about 15 amino acid residues of any of the foregoing.

92. The fusion protein of Claim 91 wherein the fusion protein transports iron across a cell membrane or an artificial cell membrane system.

20

93. An isolated immunogenic polypeptide, the amino acid sequence of which comprises at least 19 consecutive amino acid residues of SEQ ID NO:6.

94. An isolated polypeptide comprising at least 10 amino acid residues of a cytoplasmic or extracellular domain of human Ferroportin1.

95. An isolated polypeptide comprising the amino acid sequence SEQ ID NO:6, or SEQ ID NO:6 with at least one conservative amino acid substitution.

96. An isolated polypeptide, the amino acid sequence of which comprises a sequence at least 80% identical to SEQ ID NO:6.

5 97. An isolated polypeptide, the amino acid sequence of which comprises a sequence at least 80% similar to SEQ ID NO:6.

98. An isolated polypeptide the amino acid sequence of which consists of SEQ ID NO:6.

99. An isolated polypeptide produced by the method of Claim 70.

10 100. An isolated polypeptide encoded by a nucleic acid molecule comprising a nucleotide sequence which encodes a naturally occurring allelic variant of a polypeptide, the polypeptide consisting of the amino acid sequence of human Ferroportin1, wherein said nucleic acid molecule hybridizes to a complement of a nucleic acid molecule consisting of SEQ ID NO:6 under high stringency conditions.

15 101. A fusion protein comprising a polypeptide selected from the group consisting of:

20 a) a naturally occurring allelic variant of a polypeptide, the polypeptide consisting of the amino acid sequence of human Ferroportin1 in SEQ ID NO:6;

b) a polypeptide consisting of an amino acid sequence which is at least 95% identical to the amino acid sequence of SEQ ID NO:6;

c) a polypeptide consisting of amino acid sequence SEQ ID NO:6; and

d) a polypeptide comprising a contiguous portion of at least about 15 amino acid residues of any of the foregoing.

102. The fusion protein of Claim 101 wherein the fusion protein transports iron across a cell membrane or an artificial cell membrane system.

5 103. A method for eliciting an immune response in an animal, said method comprising introducing into the animal a composition comprising a polypeptide comprising at least 19 consecutive amino acid residues of SEQ ID NO:2.

10 104. A method for eliciting an immune response in an animal, said method comprising introducing into the animal a composition comprising a polypeptide comprising at least 19 consecutive amino acid residues of SEQ ID NO:4.

105. A method for eliciting an immune response in an animal, said method comprising introducing into the animal a composition comprising a polypeptide comprising at least 19 consecutive amino acid residues of SEQ ID NO:6.

15 106. Antibodies that bind specifically to a Ferroportin1 protein, where antibodies include single-chain antibodies, chimeric antibodies and immunologically active fragments of antibodies.

107. A method for producing antibodies, said method comprising introducing into an animal isolated zebrafish Ferroportin1 or an immunogenic fragment of zebrafish Ferroportin1.

108. A method for producing antibodies, said method comprising introducing into a non-murine animal isolated mouse Ferroportin1 or an immunogenic fragment of mouse Ferroportin1.

109. A method for producing antibodies, said method comprising introducing into a non-human animal isolated human Ferroportin1 or an immunogenic fragment of human Ferroportin1.

110. A method for identifying an agent which binds to a protein comprising an amino acid sequence SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6, or at least 10 contiguous amino acid residues of any of the foregoing, comprising the steps of contacting the agent with the isolated protein under conditions appropriate for binding of the agent to the isolated protein, and detecting a resulting agent-protein complex.

111. A method for identifying an agent which binds to a protein, said protein encoded by a polynucleotide comprising a nucleotide sequence which encodes a naturally occurring allelic variant of a polypeptide consisting of the amino acid sequence of human Ferroportin1, wherein said polynucleotide hybridizes to a complement of a polynucleotide consisting of SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5 under high stringency conditions, comprising the steps of isolating the protein, contacting the agent with the isolated protein under conditions appropriate for binding of the agent to the isolated protein, and detecting a resulting agent-protein complex.

112. A method for identifying an agent which inhibits interaction between an isolated polypeptide, the amino acid sequence of which is at least 80% similar to SEQ

ID NO:2, SEQ ID NO:4 or SEQ ID NO:6, and a ligand of said protein, comprising:

5

- (a) combining:
 - (1) said isolated polypeptide;
 - (2) the ligand of said polypeptide; and
 - (3) a candidate agent to be assessed for its ability to inhibit interaction between said polypeptide of (1) and the ligand of (2), under conditions appropriate for interaction between said polypeptide of (1) and the ligand of (2);
- 10 (b) determining the extent to which said polypeptide of (1) and the ligand of (2) interact; and
- (c) comparing the extent determined in (b) with the extent to which interaction of said polypeptide of (1) and the ligand of (2) occurs in the absence of the candidate agent to be assessed and under the same conditions appropriate for interaction of said polypeptide of (1) with the ligand of (2);
- 15 wherein if the extent to which interaction of said polypeptide of (1) and the ligand of (2) occurs is less in the presence of the candidate agent than in the absence of the candidate agent, the candidate agent is an agent which inhibits interaction between said polypeptide and the ligand of said polypeptide.
- 20

113. The method of Claim 112 wherein (a) is performed in an artificial membrane system.

114. The method of Claim 112 wherein said isolated polypeptide is in isolated plasma membrane.

115. A method for identifying an agent which inhibits interaction between (1) an isolated protein, said protein being encoded by a polynucleotide comprising a nucleotide sequence which encodes a naturally occurring allelic variant of a polypeptide, said polypeptide consisting of the amino acid sequence of human Ferroportin1, wherein said polynucleotide hybridizes to a complement of a polynucleotide consisting of SEQ ID NO:5 under high stringency conditions and (2) a ligand of said protein, comprising:

(a) combining:

(1) said isolated protein;

(2) the ligand of said protein; and

(3) a candidate agent to be assessed for its ability to inhibit interaction between said protein of (1) and the ligand of (2), under conditions appropriate for interaction between said protein of (1) and the ligand of (2);

(b) determining the extent to which said protein of (1) and the ligand of (2) interact; and

(c) comparing the extent determined in (b) with the extent to which interaction of said protein of (1) and the ligand of (2) occurs in the absence of the candidate agent to be assessed and under the same conditions appropriate for interaction of said protein of (1) with the ligand of (2);

wherein if the extent to which interaction of said protein of (1) and the ligand of (2) occurs is less in the presence of the candidate agent than in the absence of the candidate agent, the candidate agent is an agent which inhibits interaction between said protein and the ligand of said protein.

116. A method for identifying an agent which binds to a protein encoded by a nucleic acid encoding a human Ferroportin1 comprising an amino acid sequence sharing

at least about 95% amino acid sequence identity with the amino acid sequence SEQ ID NO:6, said method comprising the steps of isolating the protein, contacting the agent with the isolated protein under conditions appropriate for binding of the agent to the isolated protein, and detecting a resulting agent-protein complex.

5

117. A method for identifying an agent which binds to a protein encoded by a nucleic acid encoding a Ferroportin1 comprising an amino acid sequence sharing at least about 95% amino acid sequence similarity with the amino acid sequence in SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6 comprising the steps of isolating the protein, contacting the agent with the isolated protein under conditions appropriate for binding of the agent to the isolated protein, and detecting a resulting agent-protein complex.

10

118. The method of Claim 117 wherein the step of contacting the agent with isolated protein is performed in an artificial membrane system.

15 119. The method of Claim 117 wherein the isolated protein is in isolated plasma membrane.

120. A method for identifying an agent which is an inhibitor of iron export by a polypeptide comprising an amino acid sequence with at least 95% identity to SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6 comprising the steps of:

20

- a) maintaining test cells expressing said polypeptide in the presence of iron and an agent to be tested as an inhibitor of iron export;
- b) measuring export of the iron from the test cells; and
- c) comparing export of the iron from the test cells with export of iron from suitable control cells;

wherein lower export of the iron from the test cells compared to export of the iron from the control cells is indicative that the agent is an inhibitor of iron export by said protein.

121. A method for identifying an agent which is an inhibitor of iron export by a polypeptide comprising an amino acid sequence with at least 95% sequence similarity to SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6, comprising the steps of:

- 5 a) maintaining test cells expressing said polypeptide in the presence of iron and an agent to be tested as an inhibitor of iron export;
- 10 b) measuring export of the iron from the test cells; and
- 15 c) comparing export of the iron from the test cells with export of iron from suitable control cells;
wherein lower export of the iron from the test cells compared to export of the iron from the control cells is indicative that the agent is an inhibitor of iron export by said polypeptide.

122. An inhibitor of iron export identified by the method of Claim 121.

123. The method of Claim 121 further comprising the steps of:

- 20 a) administering the agent to one or more test animals;
- b) measuring exogenously supplied iron in one or more samples of tissue or bodily fluid from said test animals;
- c) measuring exogenously supplied iron in one or more comparable samples of tissue or bodily fluid from suitable control animals;
- d) comparing the iron of b) with the iron of c);
whereby, lower iron in step b) than in step c) is indicative that the agent is an inhibitor of said polypeptide.

124. An inhibitor of iron export identified by the method of Claim 121.

125. A method for identifying an agent which is an enhancer of iron export by a protein, said protein encoded by a polynucleotide comprising a nucleotide sequence which encodes a naturally occurring allelic variant of a polypeptide, the polypeptide consisting of the amino acid sequence of human Ferroportin1, wherein said polynucleotide hybridizes to a complement of a polynucleotide consisting of SEQ ID NO:5 under high stringency conditions, said method comprising the steps of:

10 a) maintaining test cells containing iron and expressing said polynucleotide in the presence of an agent to be tested as an enhancer of iron export;

b) measuring export of the iron from the test cells; and

c) comparing export of the iron from the test cells with export of the iron from suitable control cells;

15 wherein greater export of the iron from the test cells compared to export of the iron from the control cells is indicative that the agent is an enhancer of iron export by said protein.

126. An enhancer of iron export identified by the method of Claim 125.

127. The method of Claim 125 further comprising the steps of:

20 a) administering the agent to one or more test animals;

b) measuring exogenously supplied iron in one or more samples of tissue or bodily fluid from said test animals;

25 c) measuring exogenously supplied iron in one or more comparable samples of tissue or bodily fluid from suitable control animals;

d) comparing the iron of b) with the iron of c);

whereby, higher iron in step b) than in step c) is indicative that the agent is an enhancer of said protein.

128. A method for identifying an agent which is an inhibitor of a protein, said protein being encoded by a nucleic acid encoding a Ferroportin1 comprising an amino acid sequence sharing at least about 95% amino acid sequence identity with the amino acid sequence in SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6, comprising the steps of:

- a) introducing into host cells one or more vectors or RNAs comprising a polynucleotide sequence expressing said protein;
- b) culturing said host cells with iron under conditions permitting uptake of iron into said host cells;
- c) culturing a first aliquot of the host cells of b) with an agent being tested as an inhibitor of said protein;
- d) culturing a second aliquot of the host cells of b) without said agent;
- e) determining, in the first and second aliquots, export of iron from the host cells;

wherein less export of iron from the first aliquot compared to the export of iron from the second aliquot is indicative that the agent is an inhibitor of said protein.

129. A method for identifying an agent which is an enhancer of a protein, said protein being encoded by a nucleic acid encoding a Ferroportin1 comprising an amino acid sequence sharing at least about 95% amino acid sequence similarity with the amino acid sequence in SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6, comprising the steps of:

- a) introducing into host cells one or more vectors or RNAs comprising a polynucleotide sequence expressing said protein;

administering to the mammal an inhibitor of Ferroportin1 iron transport function.

132. A method for treating a disease or medical disorder resulting from oxidative damage in a mammal, said method comprising administering to the mammal an inhibitor of Ferroportin1 iron transport function.

133. A method for treating iron deficiency anemia in a mammal, said method comprising administering to the mammal an enhancer of Ferroportin1 iron transport function.

CONFIDENTIAL - 1035-002

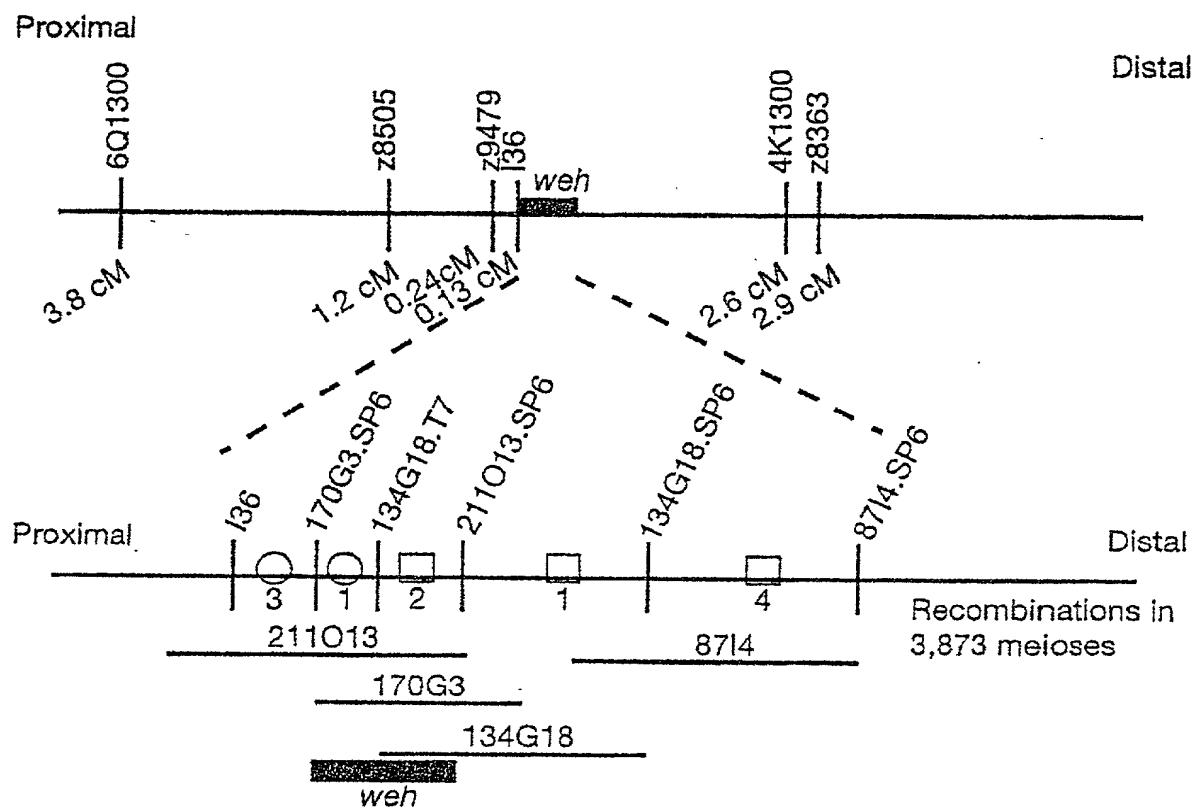


FIG. 1

Zebrafish	FPN1	M D S P A S K K P R - - - C E R F R E F F K S A K F L I Y V G H A L I S T W G D R - 37
Human	FPN1	M T R A K G D H N R D R I G C C G I S P E D V I L T S A K F I D Y I G H S I S T W G D R - 40
Mouse	FPN1	M T K A R D Q T H Q E G C C S I S A N G L I S T A K F I L Y I G H S I S T W G D R - 40
Zebrafish	FPN1	M W H F A V A V E L V E L Y G H S U L L T A V Y G L V V A G S V L L E G A I I G - 77
Human	FPN1	M W H F A V A V E L V E L Y G H S U L L T A V Y G L V V A G S V L L E G A I I G - 80
Mouse	FPN1	M W H F A V A V E L V E L Y G H S U L L T A V Y G L V V A G S V L L E G A I I G - 80
Zebrafish	FPN1	D W V D K S P R I K V A Q T S L V V Q N S A V I L G A L I M H V F L H Q K E L - 112
Human	FPN1	D W V D K S P R I K V A Q T S L V V Q N S A V I L G A L I M H V F L H Q K E L - 112
Mouse	FPN1	D W V D K S P R I K V A Q T S L V V Q N S A V I L G A L I M H V F L H Q K E L - 120
Zebrafish	FPN1	S S M Y D G W L I T T C Y I M V S T A N I A N L A S T A M S I T Q D R D W V V - 157
Human	FPN1	I T M Y D G W L I T T C Y I M V S T A N I A N L A S T A M S I T Q D R D W V V - 160
Mouse	FPN1	I T M Y D G W L I T T C Y I M V S T A N I A N L A S T A M S I T Q D R D W V V - 160
Zebrafish	FPN1	V V A G D D R S K L A D M H A T V R I D Q O L T H I A P H I L V G Q I M A F G S - 197
Human	FPN1	V V A G D D R S K L A D M H A T V R I D Q O L T H I A P H I L V G Q I M A F G S - 200
Mouse	FPN1	V V A G D D R S K L A D M H A T V R I D Q O L T H I A P H I L V G Q I M A F G S - 200
Zebrafish	FPN1	H F E G C O F I S G W N L F S M C L E Y F L D K V Y Q K T P A L A F R A G Q K - 237
Human	FPN1	P V I G C G F I S G W N L V S H C V E Y V I L W K V Y Q K T P A L A F R A G Q K - 240
Mouse	FPN1	P V I G C G F I S G W N L V S H C V E Y V I L W K V Y Q K T P A L A F R A G Q K - 240
Zebrafish	FPN1	D S D D Q E L K H I N T Q K E I G N T E S I V P A S Q U M T E S S - - - - E - 271
Human	FPN1	E - E - E T E K Q O G N L H K D T E P K - - P I L E G T H I L M G V K D S I N T R E L E - 271
Mouse	FPN1	V - E L E S E E K Q L T S P K D T E P K - - P I L E G T H I L M G V K D S I N T R E L E - 271
Zebrafish	FPN1	P K K O T G C C Y Q M A E P I R T F K D G W V A Y Y H Q S I F F A G M S L A F L - 311
Human	FPN1	H E Q E P Q C A S Q M A E P I R T F K D G W V A Y Y H Q S I F F A G M S L A F L - 317
Mouse	FPN1	C E Q E P Q C A S Q M A E P I R T F K D G W V A Y Y H Q S I F F A G M S L A F L - 317
Zebrafish	FPN1	Y M T V I E G D C I T G Y A T T Q G I L S G I S T I S I L M G A S A I T G I M G - 351
Human	FPN1	Y M T V I E G D C I T G Y A T T Q G I L S G I S T I S I L M G A S A I T G I M G - 357
Mouse	FPN1	Y M T V I E G D C I T G Y A T T Q G I L S G I S T I S I L M G A S A I T G I M G - 357
Zebrafish	FPN1	V A F T W I R K K K C G I L I R T G F I A G V T Q E S E T I L C V A S V F A P G S P - 391
Human	FPN1	V A F T W I R K K K C G I L I R T G F I A G V T Q E S E T I L C V A S V F A P G S P - 391
Mouse	FPN1	V A F T W I R K K K C G I L I R T G F I A G V T Q E S E T I L C V A S V F A P G S P - 397
Zebrafish	FPN1	F D L S V S P E K E V L R H L P G D S G I S L R E S - - - P T F I P T T E P P I - 427
Human	FPN1	F D L S V S P E K E V L R H L P G D S G I S L R E S - - - P T F I P T T E P P I - 427
Mouse	FPN1	F D L S V S P E K E D - R S R A P I Q G E S I T P T - K I P E - - I T T E I Y H S - 436
Zebrafish	FPN1	- - Q A N V T V F E E A P P E S Y M I S V G G E L E A G V I A R Y G L W S R D - 464
Human	FPN1	I N G S N S A N I V F E T S P E S V P T I S V S V L E A G V I A R Y G L W S R D - 473
Mouse	FPN1	I N M S N R - - - W H E M S T K P I P E V S V S V L E A G V I A R Y G L W S R D - 472
Zebrafish	FPN1	L T V T Q L I Q E H V I E S E R G V I T N G Y Q R S M N Y L L D I L H F I M V I L - 504
Human	FPN1	L T V T Q L I Q E H V I E S E R G V I T N G Y Q R S M N Y L L D I L H F I M V I L - 513
Mouse	FPN1	L T V T Q L I Q E H V I E S E R G V I T N G Y Q R S M N Y L L D I L H F I M V I L - 512
Zebrafish	FPN1	A P H P E A F G I L E V I T S V S V A M G H M M Y F R F A Y K S U G S R Y L E L F - 544
Human	FPN1	A P H P E A F G I L E V I T S V S V A M G H M M Y F R F A Y K S U G S R Y L E L F - 552
Mouse	FPN1	A P H P E A F G I L E V I T S V S V A M G H M M Y F R F A Y K S U G S R Y L E L F - 551
Zebrafish	FPN1	C S P I E Q K P D P R I - P S L P R S V - 562
Human	FPN1	C G P D A K I E V R K I E N Q A H T S V V - 571
Mouse	FPN1	C G P D E E K V T D E N Q P R T S V V - 579

FIG. 2

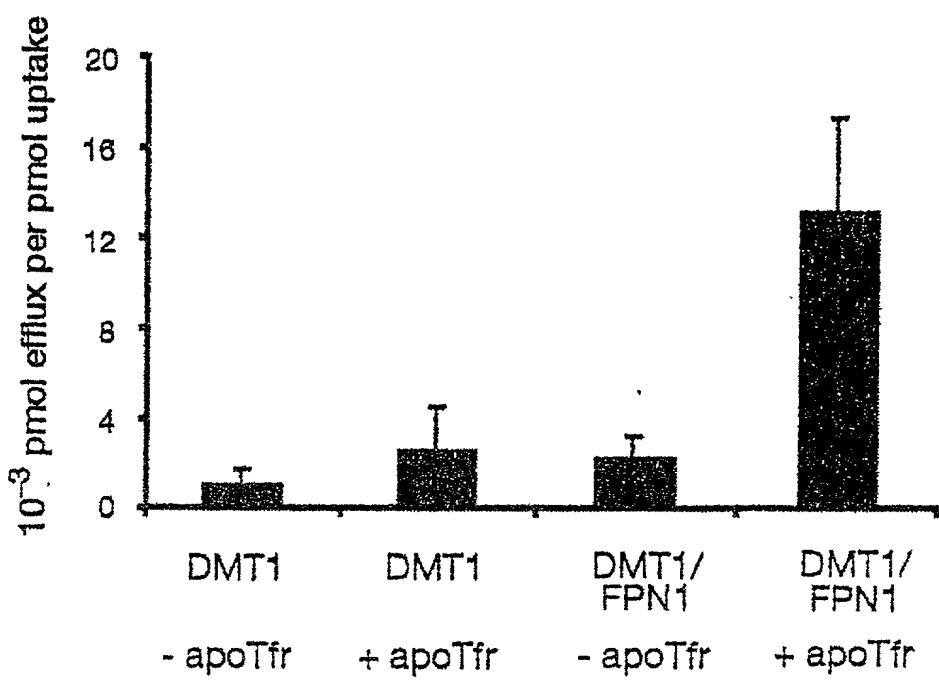


FIG. 3

Date: November 17, 2000

EXPRESS MAIL LABEL NO. EL552576675US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Leonard I. Zon and Adriana Donovan

Title: FERROPORTIN1 NUCLEIC ACIDS AND PROTEINS

TRANSMITTAL OF SEQUENCE LISTING IN COMPUTER READABLE FORM
IN COMPLIANCE WITH 37 C.F.R. §§1.821(e) AND (f)

Box Sequence
Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Transmitted herewith is a copy of the "Sequence Listing" in computer readable form as required by 37 C.F.R. §1.821(e). As required by 37 C.F.R. §1.821(f), Applicants' Attorney hereby states that the content of the "Sequence Listing" in paper form and of the computer readable form of the "Sequence Listing" are the same.

Respectfully submitted,

HAMILTON, BROOK, SMITH & REYNOLDS, P.C.

By Carol A. Egner
Carol A. Egner
Registration No. 38,866
Telephone (781) 861-6240
Facsimile (781) 861-9540

Lexington, Massachusetts 02421-4799

Date: November 17, 2000

SEQUENCE LISTING

<110> Zon, Leonard I.
Donovan, Adriana

<120> FERROPORTINI NUCLEIC ACIDS AND PROTEINS

<130> 1242.1035-002

<150> US 60/133,382
<151> 1999-05-10

<150> US 09/567,672
<151> 2000-05-09

<160> 16

<170> FastSEQ for Windows Version 4.0

<210> 1
<211> 3773
<212> DNA
<213> Danio rerio

<220>
<221> CDS
<222> (238) ... (1926)

<400> 1
acgaggtgcg agcggctctg gccatttcgg gaattatatg ttttattca catagttgtt 60
ctagaaagg tatttctctc cgacttcagc tacagtata gctaagttt gagaggagaa 120
aaggagata ttctgtgattt gcgcaggaat atatttgcag cgaggattta ctttgcgg 180
gccttacaaa ggagttcaaa tcccgccgag aaaaaaaca atcgataaaaa acgcaca atg 240
Met
1

gac agc cct gca tca aag aaa cct cgc tgt gag agg ttc cgc gaa ttc 288
Asp Ser Pro Ala Ser Lys Lys Pro Arg Cys Glu Arg Phe Arg Glu Phe
5 10 15

ttc aag tct gca aaa ttc ctc att tac gtc gga cat gcc ctc tcg aca 336
Phe Lys Ser Ala Lys Phe Leu Ile Tyr Val Gly His Ala Leu Ser Thr
20 25 30

tgg ggg gat cgg atg tgg aat ttt gct gtg gct gtg ttt ctg gtg gag 384
Trp Gly Asp Arg Met Trp Asn Phe Ala Val Ala Val Phe Leu Val Glu
35 40 45

ctg tat ggc aat agt tta ctc ctg aca gcc gtg tat gga ctg gtg gtc 432
Leu Tyr Gly Asn Ser Leu Leu Leu Thr Ala Val Tyr Gly Leu Val Val
50 55 60 65

gcg ggc tcc gtg ctc tta ctg ggc gct att att ggt gac tgg gtt gac		480	
Ala Gly Ser Val Leu Leu Leu Gly Ala Ile Ile Gly Asp Trp Val Asp			
70	75	80	
aaa aac ccc aga ttg aaa gtg gca cag acg tct ttg gtt gtc cag aac		528	
Lys Asn Pro Arg Leu Lys Val Ala Gln Thr Ser Leu Val Val Gln Asn			
85	90	95	
agt gct gtc att ctc tgt ggt gcc ctt ttg atg gct gtt ttc cag ttc		576	
Ser Ala Val Ile Leu Cys Gly Ala Leu Leu Met Ala Val Phe Gln Phe			
100	105	110	
aaa caa cag ctt tct agc atg tat gat gga tgg ttg ctg aca aca tgc		624	
Lys Gln Gln Leu Ser Ser Met Tyr Asp Gly Trp Leu Leu Thr Thr Cys			
115	120	125	
tac ata atg gtc atc tcc att gct aat atc gct aac ctg gcc agc aca		672	
Tyr Ile Met Val Ile Ser Ile Ala Asn Ile Ala Asn Leu Ala Ser Thr			
130	135	140	145
gct atg tcc atc acc atc caa aga gac tgg gtt gtg gtt gct gga		720	
Ala Met Ser Ile Thr Ile Gln Arg Asp Trp Val Val Val Ala Gly			
150	155	160	
gat gat cgg agc aaa ttg gca gat atg aat gca act gtc aga ata att		768	
Asp Asp Arg Ser Lys Leu Ala Asp Met Asn Ala Thr Val Arg Ile Ile			
165	170	175	
gac cag ttg acc aac att ctg gca ccg atg ctt gtg ggc cag atc atg		816	
Asp Gln Leu Thr Asn Ile Leu Ala Pro Met Leu Val Gly Gln Ile Met			
180	185	190	
gca ttt ggc tca cac ttc att ggc tgt ggt ttt atc tcg ggc tgg aac		864	
Ala Phe Gly Ser His Phe Ile Gly Cys Gly Phe Ile Ser Gly Trp Asn			
195	200	205	
ttg ttc tcc atg tgc ctg gag tat ttc ctg ctt tgg aaa gtt tat cag		912	
Leu Phe Ser Met Cys Leu Glu Tyr Phe Leu Leu Trp Lys Val Tyr Gln			
210	215	220	225
aaa act cca gcg ctt gcc ttt aag gca gga cag aag gat agc gat gac		960	
Lys Thr Pro Ala Leu Ala Phe Lys Ala Gly Gln Lys Asp Ser Asp Asp			
230	235	240	
caa gag ctg aaa cac ctc aac ata caa aaa gaa att gga aac act gaa		1008	
Gln Glu Leu Lys His Leu Asn Ile Gln Lys Glu Ile Gly Asn Thr Glu			
245	250	255	
agc ccg gtc gaa gcc tcc caa ctg atg act gaa agc tcc gag ccc aag		1056	
Ser Pro Val Glu Ala Ser Gln Leu Met Thr Glu Ser Ser Glu Pro Lys			
260	265	270	
aag gac acc ggc tgc tgc tac caa atg gca gag ccc atc cgt acc ttt		1104	
Lys Asp Thr Gly Cys Cys Tyr Gln Met Ala Glu Pro Ile Arg Thr Phe			
275	280	285	

aaa gat ggc tgg gta gcc tac tac aat caa tcc atc ttc ttc gcc ggc Lys Asp Gly Trp Val Ala Tyr Tyr Asn Gln Ser Ile Phe Phe Ala Gly 290 295 300 305	1152
atg tct ctg gct ttc cta tac atg acc gtt ttg ggc ttc gac tgc atc Met Ser Leu Ala Phe Leu Tyr Met Thr Val Leu Gly Phe Asp Cys Ile 310 315 320	1200
acc aca ggc tat gca tac act cag ggc ctg aat ggc tct gtg ctc agt Thr Thr Gly Tyr Ala Tyr Thr Gln Gly Leu Asn Gly Ser Val Leu Ser 325 330 335	1248
ctc ctc atg gga gcc tca gct gta tct ggg atc tgt ggg aca gtg gcc Leu Leu Met Gly Ala Ser Ala Val Ser Gly Ile Cys Gly Thr Val Ala 340 345 350	1296
ttc acc tgg atc cga aag aag tgc ggc ctc atc agg acg ggc ttc att Phe Thr Trp Ile Arg Lys Lys Cys Gly Leu Ile Arg Thr Gly Phe Ile 355 360 365	1344
gct gga gtc acc cag ctg tcc tgc ctc acg ctg tgt gta gca tct gtc Ala Gly Val Thr Gln Leu Ser Cys Leu Thr Leu Cys Val Ala Ser Val 370 375 380 385	1392
ttc gcc cct ggt agc cct ttc gat ctc agc gtc tcg ccc ttc aaa gag Phe Ala Pro Gly Ser Pro Phe Asp Leu Ser Val Ser Pro Phe Lys Glu 390 395 400	1440
gtc tta aga cat ctg ttt gga gac agc ggc tcg ctg cgt gag agt cct Val Leu Arg His Leu Phe Gly Asp Ser Gly Ser Leu Arg Glu Ser Pro 405 410 415	1488
aca ttc att cct aca act gaa ccc ccg att cag gcc aac gtc acc gtt Thr Phe Ile Pro Thr Thr Glu Pro Pro Ile Gln Ala Asn Val Thr Val 420 425 430	1536
ttt gag gaa gcc ccc cca gta gag tcc tac atg tct gtt ggg ctt ctc Phe Glu Ala Pro Pro Val Glu Ser Tyr Met Ser Val Gly Leu Leu 435 440 445	1584
ttt gcc ggt gtt att gct aga gtt ggt ctt tgg tcc ttc gac ttg Phe Ala Gly Val Ile Ala Ala Arg Val Gly Leu Trp Ser Phe Asp Leu 450 455 460 465	1632
acc gtg acc caa ctg atc caa gag aat gtg att gag tcc gag aga gga Thr Val Thr Gln Leu Ile Gln Glu Asn Val Ile Glu Ser Glu Arg Gly 470 475 480	1680
gtc atc aat ggc gtc cag aac tcc atg aat tat ctt ctc gat ctc ctg Val Ile Asn Gly Val Gln Asn Ser Met Asn Tyr Leu Leu Asp Leu Leu 485 490 495	1728

cac ttc atc atg gtc atc ctt gca cca aat cct gaa gcc ttt ggt ctt	1776
His Phe Ile Met Val Ile Leu Ala Pro Asn Pro Glu Ala Phe Gly Leu	
500	505
510	
ctt gta atc atc tcc gtt tcc ttc gtg gct atg gga cat atg atg tat	1824
Leu Val Ile Ile Ser Val Ser Phe Val Ala Met Gly His Met Met Tyr	
515	520
525	
ttc agg ttt gct tat aaa agc ctt gga agc cga ctc ttc ctg ttc tgt	1872
Phe Arg Phe Ala Tyr Lys Ser Leu Gly Ser Arg Leu Phe Leu Phe Cys	
530	535
540	545
tca ccc gag cag aag cca gat ccc aac att ccc tca ctt cca aac tct	1920
Ser Pro Glu Gln Lys Pro Asp Pro Asn Ile Pro Ser Leu Pro Asn Ser	
550	555
560	
gta tag cttttaaag agaccgtagg ccatttctac aagagcgtgg ctgctgtgt	1976
Val *	

tcttcagaa ctttgcagg atccatctg tttactaac atgcatgctt ttgctgctt	2036
cagtgtgtg cattgagtaa atatctctg ccataggcta aaataacaaa gagaaggagc	2096
tcttccttagc atagcatact tcacttctca tatcatgttc aaggtgctgt aaaaatgc	2156
tagaagcaac cgtaggagga aatatataca tggaaactac ggttctatca tgcttaatg	2216
actttgtaa gagctccaaa gcaaaaatta gcatatttat tctactttt cgtatttat	2276
tgtttttttt tttcaacttt atggcgttag ttaacctca gactggttat gacagtttt	2336
caatgtgctc tacttatgtat agttagttt tgtaatgtt gtcccttctt ccaaggctt	2396
gttaaagtct cttaatagc tattaagagt ggcctagtt tacattcagg taaggctata	2456
taatgcctat atatttatat acacgtgtac tcaatattttt ttatctcagc ttccgggt	2516
ctacgttgtt tcaactctt tggaaagccca tgcaggcggt ttatacatgt aaccaaagt	2576
ggttttttt ggcacatcgt ggaagtgggg gaattggcggt ttttttatcg tggtaaacat	2636
tccatattat tattattacc ggtgtatga tttctggag atttaggcgc tgataggctc	2696
cccatcgccag caagagattt tagcgttagg tattttgtgtt cctgtttgtt ttgaaagt	2756
tttgcaca taatttttttgc aaagattgtt acacatgcac ttatcatgtat	2816
taatatacgt tttccattac gaaacaagcg caacaagccc tcaggttata cgatatttgc	2876
acaatacaca aaacctgtcg ccgaagttca cggccaggca gggaaatctga tatttttaca	2936
tgcaaattta ttcaaaatg ggattttcaa agtacattaa cctcaaactt catgattttt	2996
accccttat ataagacacc acacccatca cgctaatcta gatttctat aataaaaaat	3056
aaaggttaca gactgttcta ttagctgaga tgaaagccac aatcatagaa gtactactaa	3116
catcctttta aaaccacagc tggctcgaca tagatata gatataatata tatgagggt	3176
tttataatag ttgtgtataa ttgatgttgg acaccagcgga gaatccacca tatgcacaga	3236
acagagaagg gattattgag tccagtggtt gaacggctgg tttgcagcgc agctggttcc	3296
aaacacaggt gccaagtcac acttgacttg ctaagttac gttttcttta atgtgtgaga	3356
actacttcat gaggccccaa cgaacacact gtcagttttt cattgtgtca gtcttctgt	3416
gaatgtgaag ctttattttac atctgtaaaa tatttttttata tattttttatgt ttgactagtt	3476
ttgtttcaat cgggtttatc ctcctttgttta aggccacaga tttcccccctt ttagacaaga	3536
gaagtaaaaca catttgcataa aaattgtact ttcgacaccc agttgaatgt aacagaagaa	3596
cctagattat tctttatata agcatattta ttctgttcat gtttgggtggc atatttgcaa	3656
taattgtgtt tcacactcca tcgcagtggtt aggattatag aacttttagtc ttgtattgt	3716
tctcacttcg actgaaataa acagattttgtt atctaaaaaaa aaaaaaaaaa aaaaaaaaaa	3773

<210> 2
 <211> 562
 <212> PRT
 <213> Danio rerio

<400> 2
 Met Asp Ser Pro Ala Ser Lys Lys Pro Arg Cys Glu Arg Phe Arg Glu
 1 5 10 15
 Phe Phe Lys Ser Ala Lys Phe Leu Ile Tyr Val Gly His Ala Leu Ser
 20 25 30
 Thr Trp Gly Asp Arg Met Trp Asn Phe Ala Val Ala Val Phe Leu Val
 35 40 45
 Glu Leu Tyr Gly Asn Ser Leu Leu Leu Thr Ala Val Tyr Gly Leu Val
 50 55 60
 Val Ala Gly Ser Val Leu Leu Leu Gly Ala Ile Ile Gly Asp Trp Val
 65 70 75 80
 Asp Lys Asn Pro Arg Leu Lys Val Ala Gln Thr Ser Leu Val Val Gln
 85 90 95
 Asn Ser Ala Val Ile Leu Cys Gly Ala Leu Leu Met Ala Val Phe Gln
 100 105 110
 Phe Lys Gln Gln Leu Ser Ser Met Tyr Asp Gly Trp Leu Leu Thr Thr
 115 120 125
 Cys Tyr Ile Met Val Ile Ser Ile Ala Asn Ile Ala Asn Leu Ala Ser
 130 135 140
 Thr Ala Met Ser Ile Thr Ile Gln Arg Asp Trp Val Val Val Ala
 145 150 155 160
 Gly Asp Asp Arg Ser Lys Leu Ala Asp Met Asn Ala Thr Val Arg Ile
 165 170 175
 Ile Asp Gln Leu Thr Asn Ile Leu Ala Pro Met Leu Val Gly Gln Ile
 180 185 190
 Met Ala Phe Gly Ser His Phe Ile Gly Cys Gly Phe Ile Ser Gly Trp
 195 200 205
 Asn Leu Phe Ser Met Cys Leu Glu Tyr Phe Leu Leu Trp Lys Val Tyr
 210 215 220
 Gln Lys Thr Pro Ala Leu Ala Phe Lys Ala Gly Gln Lys Asp Ser Asp
 225 230 235 240
 Asp Gln Glu Leu Lys His Leu Asn Ile Gln Lys Glu Ile Gly Asn Thr
 245 250 255
 Glu Ser Pro Val Glu Ala Ser Gln Leu Met Thr Glu Ser Ser Glu Pro
 260 265 270
 Lys Lys Asp Thr Gly Cys Cys Tyr Gln Met Ala Glu Pro Ile Arg Thr
 275 280 285
 Phe Lys Asp Gly Trp Val Ala Tyr Tyr Asn Gln Ser Ile Phe Phe Ala
 290 295 300
 Gly Met Ser Leu Ala Phe Leu Tyr Met Thr Val Leu Gly Phe Asp Cys
 305 310 315 320
 Ile Thr Thr Gly Tyr Ala Tyr Thr Gln Gly Leu Asn Gly Ser Val Leu
 325 330 335
 Ser Leu Leu Met Gly Ala Ser Ala Val Ser Gly Ile Cys Gly Thr Val
 340 345 350
 Ala Phe Thr Trp Ile Arg Lys Lys Cys Gly Leu Ile Arg Thr Gly Phe
 355 360 365
 Ile Ala Gly Val Thr Gln Leu Ser Cys Leu Thr Leu Cys Val Ala Ser
 370 375 380

Val Phe Ala Pro Gly Ser Pro Phe Asp Leu Ser Val Ser Pro Phe Lys
 385 390 395 400
 Glu Val Leu Arg His Leu Phe Gly Asp Ser Gly Ser Leu Arg Glu Ser
 405 410 415
 Pro Thr Phe Ile Pro Thr Thr Glu Pro Pro Ile Gln Ala Asn Val Thr
 420 425 430
 Val Phe Glu Ala Pro Pro Val Glu Ser Tyr Met Ser Val Gly Leu
 435 440 445
 Leu Phe Ala Gly Val Ile Ala Ala Arg Val Gly Leu Trp Ser Phe Asp
 450 455 460
 Leu Thr Val Thr Gln Leu Ile Gln Glu Asn Val Ile Glu Ser Glu Arg
 465 470 475 480
 Gly Val Ile Asn Gly Val Gln Asn Ser Met Asn Tyr Leu Leu Asp Leu
 485 490 495
 Leu His Phe Ile Met Val Ile Leu Ala Pro Asn Pro Glu Ala Phe Gly
 500 505 510
 Leu Leu Val Ile Ile Ser Val Ser Phe Val Ala Met Gly His Met Met
 515 520 525
 Tyr Phe Arg Phe Ala Tyr Lys Ser Leu Gly Ser Arg Leu Phe Leu Phe
 530 535 540
 Cys Ser Pro Glu Gln Lys Pro Asp Pro Asn Ile Pro Ser Leu Pro Asn
 545 550 555 560
 Ser Val

<210> 3
 <211> 2130
 <212> DNA
 <213> Mus musculus

<220>
 <221> CDS
 <222> (298) ... (2010)

<400> 3
 agagcaggct ggggtctcc tgccggccgggt ggatccctcca acccgctccc ataaggcttt 60
 ggctttccaa cttcagctac agtgttagct aagtttgaa agaagacaaa aagaagaccc 120
 cgtgacagct ttgctgttgt tgtttgcctt agttgtcctt tggggtcttt cgccataagg 180
 ctgttgtgct tatactggtg ctatctcgg ttcccttcac tcctgtgaac aagctcccg 240
 gcaagagcag ctaaagctac cagcatcaga acaaacaagg ggagaacgccc tggtgtc atg 300
 Met
 1

acc aag gca aga gat caa acc cat cag gaa gga tgc tgt gga tcc tta 348
 Thr Lys Ala Arg Asp Gln Thr His Gln Glu Gly Cys Cys Gly Ser Leu
 5 10 15

gca aac tac ctg acc tca gca aaa ttc ctc ctc tac ctt ggc cac tct 396
 Ala Asn Tyr Leu Thr Ser Ala Lys Phe Leu Leu Tyr Leu Gly His Ser
 20 25 30

ctc tcc act tgg ggg gat cgg atg tgg cac ttt gca gtg tct gtg ttt 444
 Leu Ser Thr Trp Gly Asp Arg Met Trp His Phe Ala Val Ser Val Phe
 35 40 45

ctg gtg gaa ctc tat gga aac agc ctt ctc ttg aca gct gtc tat gga	492
Leu Val Glu Leu Tyr Gly Asn Ser Leu Leu Leu Thr Ala Val Tyr Gly	
50 55 60 65	
ctg gtg gtc gca ggc tct gtt ctg gtc ctg gga gcc atc att ggt gac	540
Leu Val Val Ala Gly Ser Val Leu Val Leu Gly Ala Ile Ile Gly Asp	
70 75 80	
tgg gtg gat aag aat gcc aga ctt aaa gtg gcc cag acg tca ctg gtg	588
Trp Val Asp Lys Asn Ala Arg Leu Lys Val Ala Gln Thr Ser Leu Val	
85 90 95	
gtt cag aat gtg tcc gtc atc ctc tgc gga atc atc ctg atg atg gtt	636
Val Gln Asn Val Ser Val Ile Leu Cys Gly Ile Ile Leu Met Met Val	
100 105 110	
ttc cta cac aag aat gag ctc ctg acc atg tac cat gga tgg gtc ctt	684
Phe Leu His Lys Asn Glu Leu Leu Thr Met Tyr His Gly Trp Val Leu	
115 120 125	
act gtc tgc tac atc ctg atc act att gca aac att gca aat ttg	732
Thr Val Cys Tyr Ile Leu Ile Ile Thr Ile Ala Asn Ile Ala Asn Leu	
130 135 140 145	
gcc agt act gcc act gcg atc aca atc caa agg gac tgg att gtt gtt	780
Ala Ser Thr Ala Thr Ala Ile Thr Ile Gln Arg Asp Trp Ile Val Val	
150 155 160	
gtg gca gga gaa aac agg agc aga tta gca gac atg aat gct acc att	828
Val Ala Gly Glu Asn Arg Ser Arg Leu Ala Asp Met Asn Ala Thr Ile	
165 170 175	
aga agg att gac cag cta acc aac atc ctg gcc ccc atg gct gtc ggc	876
Arg Arg Ile Asp Gln Leu Thr Asn Ile Leu Ala Pro Met Ala Val Gly	
180 185 190	
cag att atg aca ttt ggt tct cca gtc att ggc tgt ggt ttt att tcc	924
Gln Ile Met Thr Phe Gly Ser Pro Val Ile Gly Cys Gly Phe Ile Ser	
195 200 205	
ggt tgg aat ttg gtg tcc atg tgt gtg gag tac ttc ttg ctc tgg aag	972
Gly Trp Asn Leu Val Ser Met Cys Val Glu Tyr Phe Leu Leu Trp Lys	
210 215 220 225	
gtt tac cag aag acc cct gct ctg gct gta aaa gct gct ctc aag gta	1020
Val Tyr Gln Lys Thr Pro Ala Leu Ala Val Lys Ala Ala Leu Lys Val	
230 235 240	
gag gag tca gaa ctg aag cag ctg acc tca cct aaa gat act gag cca	1068
Glu Glu Ser Glu Leu Lys Gln Leu Thr Ser Pro Lys Asp Thr Glu Pro	
245 250 255	

aaa cct ttg gag gga act cat cta atg ggt gag aaa gac tcc aac atc Lys Pro Leu Glu Gly Thr His Leu Met Gly Glu Lys Asp Ser Asn Ile 260 265 270	1116
cgt gaa ctt gaa tgt gaa caa gag ccc acc tgt gcc tcc cag atg gca Arg Glu Leu Glu Cys Glu Gln Glu Pro Thr Cys Ala Ser Gln Met Ala 275 280 285	1164
gag ccc ttc cgc act ttc cga gat gga tgg gtc tcc tac tat aac cag Glu Pro Phe Arg Thr Phe Arg Asp Gly Trp Val Ser Tyr Tyr Asn Gln 290 295 300 305	1212
cca gtg ttt ctg gct ggc atg ggc ctg gct ttc ctc tat atg aca gtc Pro Val Phe Leu Ala Gly Met Gly Leu Ala Phe Leu Tyr Met Thr Val 310 315 320	1260
ctg ggc ttt gac tgt atc act aca ggg tac gcc tac act cag ggg ctg Leu Gly Phe Asp Cys Ile Thr Thr Gly Tyr Ala Tyr Thr Gln Gly Leu 325 330 335	1308
agt gga tcc atc ctt agt att ttg atg gga gca tca gca ata act gga Ser Gly Ser Ile Leu Ser Ile Leu Met Gly Ala Ser Ala Ile Thr Gly 340 345 350	1356
ata atg gga act gtg gcc ttc acc tgg cta cgt cga aaa tgt ggc ctt Ile Met Gly Thr Val Ala Phe Thr Trp Leu Arg Arg Lys Cys Gly Leu 355 360 365	1404
gtt cgg act ggt cta ttc tca gga cta gcc cag ctt tcc tgt tta atc Val Arg Thr Gly Leu Phe Ser Gly Leu Ala Gln Leu Ser Cys Leu Ile 370 375 380 385	1452
ttg tgt gtg atc tcc gta ttc atg cct gga agc ccc ttg gac ctg tct Leu Cys Val Ile Ser Val Phe Met Pro Gly Ser Pro Leu Asp Leu Ser 390 395 400	1500
gtt tct cca ttt gaa gat atc cgt tct agg ttt gtg aat gtg gag cca Val Ser Pro Phe Glu Asp Ile Arg Ser Arg Phe Val Asn Val Glu Pro 405 410 415	1548
gtg tcc cca act acc aaa ata cct gag acc gtc ttt aca aca gaa atg Val Ser Pro Thr Thr Lys Ile Pro Glu Thr Val Phe Thr Thr Glu Met 420 425 430	1596
cat atg tcc aac atg tct aat gtc cat gag atg agt act aaa ccc atc His Met Ser Asn Met Ser Asn Val His Glu Met Ser Thr Lys Pro Ile 435 440 445	1644
ccc ata gtc tct gtc agc ctg ttt gca gga gtc att gct gct aga Pro Ile Val Ser Val Ser Leu Leu Phe Ala Gly Val Ile Ala Ala Arg 450 455 460 465	1692
atc ggt ctt tgg tcc ttt gat ttg acg gtg aca cag ttg ctg caa gaa Ile Gly Leu Trp Ser Phe Asp Leu Thr Val Thr Gln Leu Leu Gln Glu 470 475 480	1740

aat gta att gaa tct gaa aga ggc att atc aat ggt gtg cag aac tcc		1788	
Asn Val Ile Glu Ser Glu Arg Gly Ile Ile Asn Gly Val Gln Asn Ser			
485	490	495	
atg aac tac ctt ctt gac ctt ctg cat ttc atc atg gtc atc ttg gcc		1836	
Met Asn Tyr Leu Leu Asp Leu Leu His Phe Ile Met Val Ile Leu Ala			
500	505	510	
cca aat cct gaa gct ttt ggc ttg ctg gta ttg att tca gtc tcc ttt		1884	
Pro Asn Pro Glu Ala Phe Gly Leu Leu Val Leu Ile Ser Val Ser Phe			
515	520	525	
gtg gca atg gga cat ctt atg tat ttc cga ttt gcc cag aag act ctg		1932	
Val Ala Met Gly His Leu Met Tyr Phe Arg Phe Ala Gln Lys Thr Leu			
530	535	540	545
ggc aac cag att ttt gtt tgt ggt cct gat gaa aaa gaa gtt aca gat		1980	
Gly Asn Gln Ile Phe Val Cys Gly Pro Asp Glu Lys Glu Val Thr Asp			
550	555	560	
gaa aat caa ccg aat aca tct gtt gta taa aaatagttta gctgtggccc		2030	
Glu Asn Gln Pro Asn Thr Ser Val Val *			
565	570		
ctgttactag attgtggaga gcatgtgtgc ttattttgtta ctgcagaatc ccaataaatg		2090	
cctgcatttc tctccaaaaa aaaaaaaaaa aaaaaaaaaa		2130	
<210> 4			
<211> 570			
<212> PRT			
<213> Mus musculus			
<400> 4			
Met Thr Lys Ala Arg Asp Gln Thr His Gln Glu Gly Cys Cys Gly Ser			
1	5	10	15
Leu Ala Asn Tyr Leu Thr Ser Ala Lys Phe Leu Leu Tyr Leu Gly His			
20	25	30	
Ser Leu Ser Thr Trp Gly Asp Arg Met Trp His Phe Ala Val Ser Val			
35	40	45	
Phe Leu Val Glu Leu Tyr Gly Asn Ser Leu Leu Thr Ala Val Tyr			
50	55	60	
Gly Leu Val Val Ala Gly Ser Val Leu Val Leu Gly Ala Ile Ile Gly			
65	70	75	80
Asp Trp Val Asp Lys Asn Ala Arg Leu Lys Val Ala Gln Thr Ser Leu			
85	90	95	
Val Val Gln Asn Val Ser Val Ile Leu Cys Gly Ile Ile Leu Met Met			
100	105	110	
Val Phe Leu His Lys Asn Glu Leu Leu Thr Met Tyr His Gly Trp Val			
115	120	125	
Leu Thr Val Cys Tyr Ile Leu Ile Ile Thr Ile Ala Asn Ile Ala Asn			
130	135	140	
Leu Ala Ser Thr Ala Thr Ala Ile Thr Ile Gln Arg Asp Trp Ile Val			
145	150	155	160

Val Val Ala Gly Glu Asn Arg Ser Arg Leu Ala Asp Met Asn Ala Thr
 165 170 175
 Ile Arg Arg Ile Asp Gln Leu Thr Asn Ile Leu Ala Pro Met Ala Val
 180 185 190
 Gly Gln Ile Met Thr Phe Gly Ser Pro Val Ile Gly Cys Gly Phe Ile
 195 200 205
 Ser Gly Trp Asn Leu Val Ser Met Cys Val Glu Tyr Phe Leu Leu Trp
 210 215 220
 Lys Val Tyr Gln Lys Thr Pro Ala Leu Ala Val Lys Ala Ala Leu Lys
 225 230 235 240
 Val Glu Glu Ser Glu Leu Lys Gln Leu Thr Ser Pro Lys Asp Thr Glu
 245 250 255
 Pro Lys Pro Leu Glu Gly Thr His Leu Met Gly Glu Lys Asp Ser Asn
 260 265 270
 Ile Arg Glu Leu Glu Cys Glu Gln Glu Pro Thr Cys Ala Ser Gln Met
 275 280 285
 Ala Glu Pro Phe Arg Thr Phe Arg Asp Gly Trp Val Ser Tyr Tyr Asn
 290 295 300
 Gln Pro Val Phe Leu Ala Gly Met Gly Leu Ala Phe Leu Tyr Met Thr
 305 310 315 320
 Val Leu Gly Phe Asp Cys Ile Thr Thr Gly Tyr Ala Tyr Thr Gln Gly
 325 330 335
 Leu Ser Gly Ser Ile Leu Ser Ile Leu Met Gly Ala Ser Ala Ile Thr
 340 345 350
 Gly Ile Met Gly Thr Val Ala Phe Thr Trp Leu Arg Arg Lys Cys Gly
 355 360 365
 Leu Val Arg Thr Gly Leu Phe Ser Gly Leu Ala Gln Leu Ser Cys Leu
 370 375 380
 Ile Leu Cys Val Ile Ser Val Phe Met Pro Gly Ser Pro Leu Asp Leu
 385 390 395 400
 Ser Val Ser Pro Phe Glu Asp Ile Arg Ser Arg Phe Val Asn Val Glu
 405 410 415
 Pro Val Ser Pro Thr Thr Lys Ile Pro Glu Thr Val Phe Thr Thr Glu
 420 425 430
 Met His Met Ser Asn Met Ser Asn Val His Glu Met Ser Thr Lys Pro
 435 440 445
 Ile Pro Ile Val Ser Val Ser Leu Leu Phe Ala Gly Val Ile Ala Ala
 450 455 460
 Arg Ile Gly Leu Trp Ser Phe Asp Leu Thr Val Thr Gln Leu Leu Gln
 465 470 475 480
 Glu Asn Val Ile Glu Ser Glu Arg Gly Ile Ile Asn Gly Val Gln Asn
 485 490 495
 Ser Met Asn Tyr Leu Leu Asp Leu Leu His Phe Ile Met Val Ile Leu
 500 505 510
 Ala Pro Asn Pro Glu Ala Phe Gly Leu Leu Val Leu Ile Ser Val Ser
 515 520 525
 Phe Val Ala Met Gly His Leu Met Tyr Phe Arg Phe Ala Gln Lys Thr
 530 535 540
 Leu Gly Asn Gln Ile Phe Val Cys Gly Pro Asp Glu Lys Glu Val Thr
 545 550 555 560
 Asp Glu Asn Gln Pro Asn Thr Ser Val Val
 565 570

<210> 5
 <211> 2222
 <212> DNA
 <213> Homo sapiens

<220>
 <221> CDS
 <222> (305) ... (2020)

<400> 5
 agctggctca gggcgccgc taggctcgga cgacctcgctg agcctccaa accgcttcca 60
 taaggcttg ctttccaaact tcagctacag tgtagctaa gttggaaag aagaaaaaaa 120
 gaaaatccct gggcccttt tctttgttc tttgccaaag tcgtcggtgt agtcttttg 180
 cccaaggctg ttgtgtttt agaggtgcta tctccagttc ctgcactcc tgttaacaag 240
 cacctcagcg agagcagcag cagcgatagc agccgcagaa gagccagcgg ggtcgcttag 300
 tgtc atg acc agg gca gat cac aac cgc cag aga gga tgc tgt gga 349
 Met Thr Arg Ala Gly Asp His Asn Arg Gln Arg Gly Cys Cys Gly
 1 5 10 15
 tcc ttg gcc gac tac ctg acc tct gca aaa ttc ctt ctc tac ctt ggt 397
 Ser Leu Ala Asp Tyr Leu Thr Ser Ala Lys Phe Leu Leu Tyr Leu Gly
 20 25 30
 cat tct ctc tct act tgg gga gat cgg atg tgg cac ttt gcg gtg tct 445
 His Ser Leu Ser Thr Trp Gly Asp Arg Met Trp His Phe Ala Val Ser
 35 40 45
 gtg ttt ctg gta gag ctc tat gga aac agc ctc ctt ttg aca gca gtc 493
 Val Phe Leu Val Glu Leu Tyr Gly Asn Ser Leu Leu Leu Thr Ala Val
 50 55 60
 tac ggg ctg gtg gca ggg tct gtt ctg gtc ctg gga gcc atc atc 541
 Tyr Gly Leu Val Val Ala Gly Ser Val Leu Val Leu Gly Ala Ile Ile
 65 70 75
 ggt gac tgg gtg gac aag aat gct aga ctt aaa gtg gcc cag acc tcg 589
 Gly Asp Trp Val Asp Lys Asn Ala Arg Leu Lys Val Ala Gln Thr Ser
 80 85 90 95
 ctg gtg gta cag aat gtt tca gtc atc ctg tgt gga atc atc ctg atg 637
 Leu Val Val Gln Asn Val Ser Val Ile Leu Cys Gly Ile Ile Leu Met
 100 105 110
 atg gtt ttc tta cat aaa cat gag ctt ctg acc atg tac cat gga tgg 685
 Met Val Phe Leu His Lys His Glu Leu Leu Thr Met Tyr His Gly Trp
 115 120 125
 gtt ctc act tcc tgc tat atc ctg atc atc act att gca aat att gca 733
 Val Leu Thr Ser Cys Tyr Ile Leu Ile Ile Thr Ile Ala Asn Ile Ala
 130 135 140
 aat ttg gcc agt act gct act gca atc aca atc caa agg gat tgg att 781
 Asn Leu Ala Ser Thr Ala Thr Ala Ile Thr Ile Gln Arg Asp Trp Ile
 145 150 155

12/19

gtt gtt gtt gca gga gaa gac aga agc aaa cta gca aat atg aat gcc Val Val Val Ala Gly Glu Asp Arg Ser Lys Leu Ala Asn Met Asn Ala 160 165 170 175	829
aca ata cga agg att gac cag tta acc aac atc tta gcc ccc atg gct Thr Ile Arg Arg Ile Asp Gln Leu Thr Asn Ile Leu Ala Pro Met Ala 180 185 190	877
gtt ggc cag att atg aca ttt ggc tcc cca gtc atc ggc tgt ggc ttt Val Gly Gln Ile Met Thr Phe Gly Ser Pro Val Ile Gly Cys Gly Phe 195 200 205	925
att tcg gga tgg aac ttg gta tcc atg tgc gtg gag tac gtc ctg ctc Ile Ser Gly Trp Asn Leu Val Ser Met Cys Val Glu Tyr Val Leu Leu 210 215 220	973
tgg aag gtt tac cag aaa acc cca gct cta gct gtg aaa gct ggt ctt Trp Lys Val Tyr Gln Lys Thr Pro Ala Leu Ala Val Lys Ala Gly Leu 225 230 235	1021
aaa gaa gag gaa act gaa ttg aaa cag ctg aat tta cac aaa gat act Lys Glu Glu Glu Thr Glu Leu Lys Gln Leu Asn Leu His Lys Asp Thr 240 245 250 255	1069
gag cca aaa ccc ctg gag gga act cat cta atg ggt gtg aaa gac tct Glu Pro Lys Pro Leu Glu Gly Thr His Leu Met Gly Val Lys Asp Ser 260 265 270	1117
aac atc cat gag ctt gaa cat gag caa gag cct act tgt gcc tcc cag Asn Ile His Glu Leu Glu His Glu Gln Glu Pro Thr Cys Ala Ser Gln 275 280 285	1165
atg gct gag ccc ttc cgt acc ttc cga gat gga tgg gtc tcc tac tac Met Ala Glu Pro Phe Arg Thr Phe Arg Asp Gly Trp Val Ser Tyr Tyr 290 295 300	1213
aac cag cct gtg ttt ctg gct ggc atg ggt ctt gct ttc ctt tat atg Asn Gln Pro Val Phe Leu Ala Gly Met Gly Leu Ala Phe Leu Tyr Met 305 310 315	1261
act gtc ctg ggc ttt gac tgc atc acc aca ggg tac gcc tac act cag Thr Val Leu Gly Phe Asp Cys Ile Thr Thr Gly Tyr Ala Tyr Thr Gln 320 325 330 335	1309
gga ctg agt ggt tcc atc ctc agt att ttg atg gga gca tca gct ata Gly Leu Ser Gly Ser Ile Leu Ser Ile Leu Met Gly Ala Ser Ala Ile 340 345 350	1357
act gga ata atg gga act gta gct ttt act tgg cta cgt cga aaa tgt Thr Gly Ile Met Gly Thr Val Ala Phe Thr Trp Leu Arg Arg Lys Cys 355 360 365	1405
ggt ttg gtt cgg aca ggt ctg atc tca gga ttg gca cag ctt tcc tgt Gly Leu Val Arg Thr Gly Leu Ile Ser Gly Leu Ala Gln Leu Ser Cys 370 375 380	1453

ttg atc ttg tgt gtg atc tct gta ttc atg cct gga agc ccc ctg gac	1501		
Leu Ile Leu Cys Val Ile Ser Val Phe Met Pro Gly Ser Pro Leu Asp			
385	390	395	
ttg tcc gtt tct cct ttt gaa gat atc cga tca agg ttc att caa gga	1549		
Leu Ser Val Ser Pro Phe Glu Asp Ile Arg Ser Arg Phe Ile Gln Gly			
400	405	410	415
gag tca att aca cct acc aag ata cct gaa att aca act gaa ata tac	1597		
Glu Ser Ile Thr Pro Thr Lys Ile Pro Glu Ile Thr Thr Glu Ile Tyr			
420	425	430	
atg tct aat ggg tct aat tct gct aat att gtc ccg gag aca agt cct	1645		
Met Ser Asn Gly Ser Asn Ser Ala Asn Ile Val Pro Glu Thr Ser Pro			
435	440	445	
gaa tct gtg ccc ata atc tct gtc agt ctg ctg ttt gca ggc gtc att	1693		
Glu Ser Val Pro Ile Ile Ser Val Ser Leu Leu Phe Ala Gly Val Ile			
450	455	460	
gct gct aga atc ggt ctt tgg tcc ttt gat tta act gtg aca cag ttg	1741		
Ala Ala Arg Ile Gly Leu Trp Ser Phe Asp Leu Thr Val Thr Gln Leu			
465	470	475	
ctg caa gaa aat gta att gaa tct gaa aga ggc att ata aat ggt gta	1789		
Leu Gln Glu Asn Val Ile Glu Ser Glu Arg Gly Ile Ile Asn Gly Val			
480	485	490	495
cag aac tcc atg aac tat ctt ctt gat ctt ctg cat ttc atc atg gtc	1837		
Gln Asn Ser Met Asn Tyr Leu Leu Asp Leu Leu His Phe Ile Met Val			
500	505	510	
atc ctg gct cca aat cct gaa gct ttt ggc ttg ctc gta ttg att tca	1885		
Ile Leu Ala Pro Asn Pro Glu Ala Phe Gly Leu Leu Val Leu Ile Ser			
515	520	525	
gtc tcc ttt gtg gca atg ggc cac att atg tat ttc cga ttt gcc caa	1933		
Val Ser Phe Val Ala Met Gly His Ile Met Tyr Phe Arg Phe Ala Gln			
530	535	540	
aat act ctg gga aac aag ctc ttt gct tgc ggt cct gat gca aaa gaa	1981		
Asn Thr Leu Gly Asn Lys Leu Phe Ala Cys Gly Pro Asp Ala Lys Glu			
545	550	555	
gtt agg aag gaa aat caa gca aat aca tct gtt gtt tga gacagttaa	2030		
Val Arg Lys Glu Asn Gln Ala Asn Thr Ser Val Val *			
560	565	570	
ctgttgctat cctgttacta gattatata gacatgtg ctatgttacta actgcagaat	2090		
tccaaataat ggctgggtgt ttgtctgt tttaccaca gctgtgcctt gagaactaaa	2150		
agctgttttag gaaacctaag tcagcagaaa ttaactgatt aatttccctt atgttgaggc	2210		
atggaaaaaa aa	2222		

<210> 6
 <211> 571
 <212> PRT
 <213> Homo sapiens

<400> 6
 Met Thr Arg Ala Gly Asp His Asn Arg Gln Arg Gly Cys Cys Gly Ser
 1 5 10 15
 Leu Ala Asp Tyr Leu Thr Ser Ala Lys Phe Leu Leu Tyr Leu Gly His
 20 25 30
 Ser Leu Ser Thr Trp Gly Asp Arg Met Trp His Phe Ala Val Ser Val
 35 40 45
 Phe Leu Val Glu Leu Tyr Gly Asn Ser Leu Leu Leu Thr Ala Val Tyr
 50 55 60
 Gly Leu Val Val Ala Gly Ser Val Leu Val Leu Gly Ala Ile Ile Gly
 65 70 75 80
 Asp Trp Val Asp Lys Asn Ala Arg Leu Lys Val Ala Gln Thr Ser Leu
 85 90 95
 Val Val Gln Asn Val Ser Val Ile Leu Cys Gly Ile Ile Leu Met Met
 100 105 110
 Val Phe Leu His Lys His Glu Leu Leu Thr Met Tyr His Gly Trp Val
 115 120 125
 Leu Thr Ser Cys Tyr Ile Leu Ile Ile Thr Ile Ala Asn Ile Ala Asn
 130 135 140
 Leu Ala Ser Thr Ala Thr Ala Ile Thr Ile Gln Arg Asp Trp Ile Val
 145 150 155 160
 Val Val Ala Gly Glu Asp Arg Ser Lys Leu Ala Asn Met Asn Ala Thr
 165 170 175
 Ile Arg Arg Ile Asp Gln Leu Thr Asn Ile Leu Ala Pro Met Ala Val
 180 185 190
 Gly Gln Ile Met Thr Phe Gly Ser Pro Val Ile Gly Cys Gly Phe Ile
 195 200 205
 Ser Gly Trp Asn Leu Val Ser Met Cys Val Glu Tyr Val Leu Leu Trp
 210 215 220
 Lys Val Tyr Gln Lys Thr Pro Ala Leu Ala Val Lys Ala Gly Leu Lys
 225 230 235 240
 Glu Glu Glu Thr Glu Leu Lys Gln Leu Asn Leu His Lys Asp Thr Glu
 245 250 255
 Pro Lys Pro Leu Glu Gly Thr His Leu Met Gly Val Lys Asp Ser Asn
 260 265 270
 Ile His Glu Leu Glu His Glu Gln Glu Pro Thr Cys Ala Ser Gln Met
 275 280 285
 Ala Glu Pro Phe Arg Thr Phe Arg Asp Gly Trp Val Ser Tyr Tyr Asn
 290 295 300
 Gln Pro Val Phe Leu Ala Gly Met Gly Leu Ala Phe Leu Tyr Met Thr
 305 310 315 320
 Val Leu Gly Phe Asp Cys Ile Thr Thr Gly Tyr Ala Tyr Thr Gln Gly
 325 330 335
 Leu Ser Gly Ser Ile Leu Ser Ile Leu Met Gly Ala Ser Ala Ile Thr
 340 345 350
 Gly Ile Met Gly Thr Val Ala Phe Thr Trp Leu Arg Arg Lys Cys Gly
 355 360 365
 Leu Val Arg Thr Gly Leu Ile Ser Gly Leu Ala Gln Leu Ser Cys Leu
 370 375 380

Ile Leu Cys Val Ile Ser Val Phe Met Pro Gly Ser Pro Leu Asp Leu
 385 390 395 400
 Ser Val Ser Pro Phe Glu Asp Ile Arg Ser Arg Phe Ile Gln Gly Glu
 405 410 415
 Ser Ile Thr Pro Thr Lys Ile Pro Glu Ile Thr Thr Glu Ile Tyr Met
 420 425 430
 Ser Asn Gly Ser Asn Ser Ala Asn Ile Val Pro Glu Thr Ser Pro Glu
 435 440 445
 Ser Val Pro Ile Ile Ser Val Ser Leu Leu Phe Ala Gly Val Ile Ala
 450 455 460
 Ala Arg Ile Gly Leu Trp Ser Phe Asp Leu Thr Val Thr Gln Leu Leu
 465 470 475 480
 Gln Glu Asn Val Ile Glu Ser Glu Arg Gly Ile Ile Asn Gly Val Gln
 485 490 495
 Asn Ser Met Asn Tyr Leu Leu Asp Leu Leu His Phe Ile Met Val Ile
 500 505 510
 Leu Ala Pro Asn Pro Glu Ala Phe Gly Leu Leu Val Leu Ile Ser Val
 515 520 525
 Ser Phe Val Ala Met Gly His Ile Met Tyr Phe Arg Phe Ala Gln Asn
 530 535 540
 Thr Leu Gly Asn Lys Leu Phe Ala Cys Gly Pro Asp Ala Lys Glu Val
 545 550 555 560
 Arg Lys Glu Asn Gln Ala Asn Thr Ser Val Val
 565 570

<210> 7
<211> 7496
<212> DNA
<213> Homo sapiens

<400> 7
 agctggctca gggcgccgc taggctcgga cgacctgctg agcctccaa accgcttcca 60
 taaggctttg ctttccaaact tcagctacag tggtagctaa gttggaaag aaggaaaaaa 120
 gaaaatccct gggccccc ttctttgttc tttgccaaag tcgtcggtgt agtcttttg 180
 cccaaggctg ttgtgtttt agaggtgcta tctccagttc ctgcactcc tggtaacaag 240
 cacctcagcg agagcagcag cagcgatagc agccgcagaa gagccagcgg ggtcgctag 300
 tgcgtatgacc agggcgggag atcacaaccg ccagagagga tgctgtggtg agtgcgttg 360
 accgaaagca tatggtgaa acccaggtgg ggctttggag acaagcaact ctacccgagt 420
 tctggaggaa tggctctg ctgtgaacca tagcttgta aaaagatcct ttgactcata 480
 tttggtgac gttaaaggaaag aaaggaaatt cagggtgtgg gaaaagggtt ttgcacacag 540
 gcacggatgg agtagattgg gcagtttggta ttgccttggtaaaaaaagaa aaaaaacaaa 600
 ccaaccaacc cacggggaa aaaacaaacc aaacaaacca accaacccaa aaaagaatgc 660
 tggaaacaaga gtttctctc tggatgtgaa atgtgaagtt gggcagttat tgacttagtc 720
 aataactgaa tttagtgaat ggtttaagt gaaacgaaata catcggtca tagttaactt 780
 gataaaatgt acgtggtttgc tccgtccaaag tagttttaa taatcatgtt ctaatgagat 840
 caaatggata agcattctgc cctcagctca ttaagtgact accatcgctt tttctcaccc 900
 cgcctgtgtc tttgcaggat ccttggccga ctacctgacc tctgcaaat tccttctcta 960
 cttgggtcat tctctctcta cttggtaag tgagaatgca tagtcttaca acacagttgc 1020
 gcaattttt atttccttgc gttcttagcc gttgtttaaa gccaacttcc agttttgtca 1080
 agcagttaaa gaaataaaagg gggaaagagta attactgact ttgaaagtct cataatgttag 1140
 ccaggccgtg ccctttgtat aaggaagcaa cttcctgagt acaatagact agaaacgaaa 1200
 aatattccat caaaacattt tctctttca tttaaggag atcggatgtg gcactttgcg 1260
 gtgtctgtgt ttctggtaga gctctatgga aacagcctcc ttttgcacgc agtctacggg 1320
 ctgggtgtgg cagggtctgt tctggcctg ggagccatca tcggtgactg ggtggacaag 1380

aatgctagac taaaaggta gtgttgttat ataattaagc ccttttattc atgggaccac 1440
tgcctgagct acctctgtag caaaggaaac aacaaactag gagagaaaaca accagggaaat 1500
gtctgcatgc cacacttgag ggaggagggc ttagatggca ccacacctgg atggagggtc 1560
ccatggctcc cacacaaagt tggatgcct ggacattgac ctaataagatt tttttgtatc 1620
tttggctgtt cataaatttc atatgttaat gattaacctt gtacacttc tctgagaacc 1680
atgttaaaca taaaagttt gcttaactca ggcttcctaa ctgtatctt tactggagtc 1740
ccttagtgtt gatgttcctg agacagctt aacatctgtt ctttgggtac tatgtttcat 1800
gtaagagttt gtataaggaa attgaaaact aagaatagct tcaaggcaga atagttgago 1860
ctggatcaca aagagctgaa ttataaattt ttagggaaa aagaagaaat aataatatct 1920
tcatatttt tctaaggatt agtactgaaa tcatgtcatt ttatacagga aaagaaaagta 1980
atgtatcaat taaatttttca agtatataag gaaaatatgg atgatcattc agggtaaattt 2040
ttcttgaatt gctcaattga taatggccaa gaacctgacc atgcctgact tagggggatg 2100
taggttctcg ccttaatcat ctgctactga gggcagagaa aaggctacca ggtgtcttta 2160
tctgtcctta ctccagtgct ttatctatat gggcgcctca taagagagtt gccatctgtg 2220
atgaaaggaa agtttagaat ttctgtgcaaa tggcaaatag cgtagatgt caaagaaaata 2280
ccctgctgct ttattctggg caaatttttgc tggctttttt ctatttaggt aagccatatt 2340
atcagattca gcctgcccattt taggagttt taggtttttagt aacttcctct ttaacctcat 2400
acatgttatt gtttacctt aagcaacaaa gagctgaaat gtggatcatg tctatatcat 2460
actacagctc catttatgtt aaacttcaa gaagataaac taaatgaaaa ggtagtcatt 2520
atgatagact tcagtggca gagaagcttgg tggacttca tcatttggtt tgcatattta 2580
ctggctctgg gtgatccctt gggttgtatt gagagtagttt gaggcaggac tgacttcaga 2640
aaggttttct tttatctgg taataatttagt gtctgggtat taatgttatta tagtagagca 2700
attatgtgtg gataagagca gtctcagtga gccattttga tgtaatgtac actttctctc 2760
ttccctctgca cagtggccca gacctcgctg gtggtacaga atgttttagt catcctgtgt 2820
ggaatcatcc tggatgtgtt tttcttacat aaacatgagc ttctgaccat gtaccatgg 2880
tgggttctcg taagttctca atgagattttt tggcggcaga aaattgaata tctggtagtg 2940
gtaaaaggatg aaaatgtttttaa gaagcttggg tttttttggg ggaggatgg ggtgtggat 3000
aaccctatgca tctgggtgtca tattgaatct tcttggatgtat atgtggattt atattataga 3060
gttgc当地 cagtagggac ttttagaaatc tttgagccctt tttcccttcat tttattgaaa 3120
aaattaaagac aaagtgaacg ttagttttagt gcccattgtc atgcaactag aagggtgtcag 3180
aactctgact taaaatcagg tggatgttcaat tcccttcaat cattctttc aaaggcaata 3240
tttggggagaaatgttcaaa aaccaccact gtgttaacat tttataactg tattcacctg 3300
actattataa tttttgtatt atgtgtacta cagatgtatct agatgataca ggttaggaca 3360
ttatgccat tgactactgg tattcattca gttccatatac tataacgtaa aatgatttct 3420
tataaatgaa attaaaatac ttttttttac attccaccaa agactatccc aactgcctt 3480
gttttagtgac atatgtacag tggatgttcaaa tgacattata actcatatctt ttcttgcata 3540
tcttttagact tcctgtata tcctgtatcat cactattgca aatattgcaaa atttggccag 3600
tactgtact gcaatcacaa tccaaaggaa ttggatgtt gttgtgcag gagaagacag 3660
aagcaacta gcaagtaatt tggctttctc ttttaatgaa atgagcatgt taggattcac 3720
tttaaatcgg tggatgttcaaa tgaggctgtt agttgtatt tttgttctgg gtattttta 3780
agaatgataa attgaaagca tacttttttca ttaccttattt gtcagttta gtgtgtattt 3840
atctcactgt tacgaagtttta acttatagga tagctaactt ctctttatcc tacggggaa 3900
accaacattt taggaatcta tactcttggg ttacagctt gtattgtgtaaatgggcagt 3960
ctctcttga tgggtttgca cacttacctg cctcttccac cggccctcttct agatatgaat 4020
gccacaatac gaaggattga ccagtttacc aacatcttag cccccatggc tggccag 4080
attatgacat tggctcccc agtcatcgcc tggatgttcaaa tttcggtatc gaacttggta 4140
tccatgtcg tggatgtacgt cctgctctgg aagtttacc agaaaacccc agctctagct 4200
gtgaaagctg gtcttaaaga agaggaaact gaattgaaac agctgaattt acacaaaggt 4260
aaactgaaca caatgtatctc tccttttgc tctatgttca gaccttaaat gttgtgtaaag 4320
atcaaaaacta tttgtatctt gtatcaggat ttattaccag tggggccag atgaggtttaa 4380
atataatcgct ttgttagacg aggcaagagc aggcttttga ggatcttaggg aaaaactccg 4440
ggttgaatct ggtgggttta gaatgggtcc cctagccctc ttcttgcata tgacgttag 4500
ttatagaggt tcaatttttac ttgagagata gctggccaaa gtcagttcat aggactgg 4560
aaaaatgtgg gaaaaaaaag agaatgagag aatcccttgg actctgtgag gagggagttta 4620
tgtgtatcattt tggatgttcaaa tggagggag tgaggacaca aagatgggtta tttctactgg 4680

gaagaggacg ctgggcttct gggtaaacag aatctttat ccactctgca gggaccacaga 4740
 aaataaatatg ctggttgttt tttgttttt tgagacagag tctcgctctg ttggccaggc 4800
 tgaagtgcag tggcgcgatc ttggctcaact gcaagctctg cctcctgggt tcacgcccatt 4860
 ctccctgcctc agcctccaa gtagctggga ttgcaggcat ccaccaccac acccggctaa 4920
 tttttgtat ttttagtaga gacggggttt caccatgtt a cccaggatgg tcttgatctc 4980
 ctgacctcgat gatctggcccg cctcgccctc ccaaagtgc gggattacag gtgtgagcca 5040
 ccgtgcctgg ccaatacgct gtgtttttt agacaatttt aatattttat ctggtgagtt 5100
 ttcctgctgt ttactttggt gggagtataa tttctaagag caagagagag agagaaaaaaa 5160
 aagagggata gatcaatagt atttgttta ttaataaaaa atgacactt atgattattc 5220
 ctggctgga attcttagat tattgtaaa agaaaataca tattacaatg tctaaccagg 5280
 ggtacccatt gggaaaggggaa atagaaggaa aaaaagact actaataatt ggcttttatt 5340
 tctacatgtc ctccccaaaca aaataatggt atctttctt aacagataact gagccaaaac 5400
 ccctggaggg aactcatcta atgggtgtga aagactctaa catccatgag cttgaacatg 5460
 agcaagagcc tacttggcc tcccaagatgg ctgagccctt ccgtacccctc cgagatggat 5520
 gggctccta ctacaaccag cctgtgttcc tggctggcat gggctttgct ttcccttata 5580
 tgactgtcct gggctttgac tgcatcacca cagggtacgc ctacactcag ggacttgatg 5640
 gttccatcct cagtttttgc atgggagcat cagctataac tggataatg ggaactgttag 5700
 cttttacttg gctacgtcga aaatgtggtt tggttccggac aggtctgtat tcaggattgg 5760
 cacagcttc ctgtttgatc ttgtgtgtga tctctgtatt catgcctgga agccccctgg 5820
 acttgtccgt ttctccctttt gaagatatcc gatcaagggtt cattcaagga gactcaatta 5880
 cacctaccaa gataccctgaa attacaactg aaatatacat gtctaatggg tctaattctg 5940
 ctaatattgt cccggagaca agtccctgaaat ctgtgccccat aatctctgtc agtctgtgt 6000
 ttgcaggcgt cattgtctgtc agaatcggtaa agaaatctct ttttatataat taatgaacta 6060
 aagtgtctt ttgtaatgtt gtttcagaga atccattaaat aaatgatctg aaatgttccc 6120
 taaatgttaa ttaaagccaa atccacttcc acgaaattttt tattttacat atttataactt 6180
 tatatttttattt gtgttttttta ttttatagtt tggaaaacctg tatttgcattt ctttattata 6240
 tacatataact taaaacatgg ttcaaggcttggaaaataattt tttctaaatg aatatcttaa 6300
 atattacggg ggcaaaaggc ttctctagcc atatgtattt attatataatg ttgccacaca 6360
 aatggattttt atagccctgg aaggaaacat aaagacttct tacaagccaa aatttaagta 6420
 ataattaata gactgtatgat gaattatctc tgaatttgc tttttttttt 6480
 atcttgcgtat acaaaaacatg tcattttttt atttgcattt ttaatttcca ggcaagacag 6540
 ctttattttt tggcttttag aactcttagca gtaatataac aatggttttaa agtttccctta 6600
 cactttaacc ataaccattt attaggtcat ttgaaacttta aaaaataacta gtacttatac 6660
 tataatagga ttattttatgt ctctgatttcc aagttttgtt tttttttttt tgaataatca 6720
 cagaaaaaaca gaactaagaa gtttgcgtat tagacttctt tttttttttt gactgtaaaa 6780
 atcatttttattt gaggccacta ataaccatattt atttatttttattt gaaaataat tcttaaggca 6840
 agctatggat atatttaagg tgactttaaag acagtccggc taaaatgtat attttgcata 6900
 tgtcaacaga tttttatctg tgatttggaaa tttttttttt tttttttttt 6960
 aaaaaaaatatttttattatgt gttttttttt tttttttttt tttttttttt 7020
 agaaaatgtt atttgcattt aacccatgat tttttttttt tttttttttt 7080
 tttttttttt tttttttttt tttttttttt tttttttttt 7140
 ctttgcgtat ttgtttttttt tttttttttt tttttttttt 7200
 tttttttttt tttttttttt tttttttttt tttttttttt 7260
 gaaatggggat caagccaaata catctgttgc tttttttttt tttttttttt 7320
 actagatttttattt atagagccaca tttttttttt tttttttttt 7380
 gtgtttttttt tttttttttt tttttttttt tttttttttt 7440
 taagtccggc gaaatattttt tttttttttt tttttttttt 7496

<210> 8
 <211> 19
 <212> DNA
 <213> Danio rerio

<400> 8
 aaa gaa gtg cg^g cct cat c

19

<210> 9
 <211> 19
 <212> DNA
 <213> Danio rerio

<220>
 <221> mutation
 <222> 10
 <223> wehth238 allele

<400> 9
 aaa gaa gtg agg cct cat c

19

<210> 10
 <211> 19
 <212> DNA
 <213> Danio rerio

<400> 10
 gag caa att ggc agg taa g

19

<210> 11
 <211> 19
 <212> DNA
 <213> Danio rerio

<220>
 <221> mutation
 <222> 10
 <223> wehtp85c allele

<400> 11
 gag caa att tgc agg taa g

19

<210> 12
 <211> 21
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Synthetic PCR Primer

<400> 12	21
caa ctt cag cta cag tgt tag	
<210> 13	
<211> 22	
<212> DNA	
<213> Artificial Sequence	
<220>	
<223> Synthetic PCR Primer	
<400> 13	22
tta tac aac aga tgt att cgg t	
<210> 14	
<211> 21	
<212> DNA	
<213> Artificial Sequence	
<220>	
<223> Synthetic PCR Primer	
<400> 14	21
aac tgt ctc aaa caa cag atg	
<210> 15	
<211> 30	
<212> DNA	
<213> Artificial Sequence	
<220>	
<223> Synthetic PCR Primer	
<400> 15	30
ccg ctc gag aac gca caa tgg aca gcc ctg	
<210> 16	
<211> 30	
<212> DNA	
<213> Artificial Sequence	
<220>	
<223> Synthetic PCR Primer	
<400> 16	30
ccg ctc gag tac aga gtt tgg aag tga ggg	